Therapeutic Effects of Intravenous Immunoglobulin, Platelet Transfusion and B Cell Depletion on Murine Immune Thrombocytopenia (ITP)

By

Li Guo

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy (Ph.D.)
Institute of Medical Science
University of Toronto

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Doctor of Philosophy

Institute of Medical Science
University of Toronto
2016

ABSTRACT

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by an isolated thrombocytopenia and increased tendency of bleeding. The pathogenesis of ITP includes both decreased platelet production by megakaryocytes in the bone marrow and increased peripheral platelet destruction in the spleen due to antibody- and T cell-mediated immune responses against self platelet antigens. To better understand the disease and its therapies, I carried out three projects focusing on antibody-mediated ITP, T cell-mediated ITP and the relationship between the two immune pathways. In the first project, I examined the number and morphology of bone marrow megakaryocytes after induction of acute thrombocytopenia by anti-platelet antibodies with different specificities and isotypes. Most of the antibodies did not appear to alter megakaryocyte morphology or maturation within 24 hours. Moreover, although IVIg therapy successfully rescued the thrombocytopenia, it did not exhibit any significant effect on the megakaryocytes. Second, using an active ITP mouse model, I investigated the effect of allogeneic platelet transfusion therapy on T cell-mediated ITP. I found an immunosuppressive effect of the allogeneic transfusions on the T cell-mediated ITP associated with a decrease in the cytotoxic activity of CD8+ T cells in vitro and normalized megakaryocyte number/morphology in the bone marrow. Third, to investigate the relationship between B and T cells in ITP, I depleted B cells in vivo and examined the changes in T cell-mediated ITP. CD8+ T cell-mediated ITP was found ameliorated after B cell depletion therapy and was associated with a
decreased proliferation of CD8$^+$ T cells that could be rescued with interleukin (IL)-2 in vitro. This study provided a novel explanation for the therapeutic effect of B cell depletion in ITP and suggests a synergistic role between B cells and T cells in initiating the disease. In summary, this thesis revealed a lack of effect of anti-platelet antibodies on megakaryocytes suggesting that T cells may be involved in mediating bone marrow effects in ITP. Related to the T cells, it further elucidated the therapeutic effects of platelet transfusions and B cell depletion therapy on T cell-mediated ITP. These results may be of importance in decisions regarding the management of ITP.
ACKNOWLEDGEMENTS

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I am also grateful to all the lab members for their support and friendship. In particular, I thank Dr. Rukhsana Aslam and Edwin R. Speck, for their tremendous support, patient teaching, extreme kindness and loving friendship. I would also like to thank lab members Michael Kim, Dr. Anne Zufferey, Dr. Rick Kapur, Dr. Christopher G. J. McKenzie, Dr. Mark McVey and all our summer students. I will always cherish our time full of laughter and joy.

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Finally I would like to thank my parents and grandmother for their unconditional love and support that travelled half of the planet with twelve hours of time difference. I could never have accomplished my PhD without their love.

I dedicate this thesis to my mom Haiyan, dad Qiang, and my grandma Yuying.

---

Basin inscription of Tang: Truly new each day. New each and every day. Again, new each day.
(苟日新 日日新 又日新)

Confucius (551 – 479 BC), The Great Learning
CONTRIBUTIONS

Li Guo (author) solely prepared this thesis. All aspects of this body of work, including the planning, execution, analysis, and writing of all original research and publications was performed in whole or in part by the author. The following contributions by other individuals are formally acknowledged:

Dr. John W. Semple (Supervisor and Thesis Committee Member) – mentorship; laboratory resources; guidance and assistance in planning, execution, and analysis of experiments as well as manuscript/thesis preparation.

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Dr. Gerald Prud’homme (Thesis Committee Member) – mentorship; guidance in planning of experiments and interpretation of results.


Dr. Rukhsana Aslam – assistance in execution of experiments and manuscript editing for Chapter 2.

Dr. Rick Kapur – assistance in manuscript editing for Chapter 2 and Chapter 4.

Mr. Edwin R. Speck – assistance in execution of experiments.
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>B10 cells</td>
<td>CD5^+CD1d^hi B cells</td>
</tr>
<tr>
<td>Bdep</td>
<td>B cell depletion</td>
</tr>
<tr>
<td>BlyS</td>
<td>B-lymphocyte stimulator</td>
</tr>
<tr>
<td>Breg</td>
<td>regulatory B cell</td>
</tr>
<tr>
<td>CCL5, RANTES</td>
<td>Chemokine (C-C motif) ligand 5, or released upon activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>CCR</td>
<td>chemokine receptor</td>
</tr>
<tr>
<td>cDC</td>
<td>conventional dendritic cell</td>
</tr>
<tr>
<td>CDR</td>
<td>complementarity determining region</td>
</tr>
<tr>
<td>CFSE</td>
<td>5-(6)-carboxyflourescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CLEC-2</td>
<td>C-type lectin domain family 2</td>
</tr>
<tr>
<td>CPDA</td>
<td>citrate-phosphate-dextrose with adenine</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CVX</td>
<td>convulxin</td>
</tr>
<tr>
<td>CXCL4</td>
<td>chemokine (C-X-C motif) ligand 4</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DCIR2, CLEC4A4</td>
<td>dendritic cell inhibitory receptor 2</td>
</tr>
<tr>
<td>DMS</td>
<td>demarcation membrane system</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein–Barr virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunoabsorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FNAIT, NAIT, NAITP or NATP</td>
<td>fetal and neonatal alloimmune thrombocytopenia</td>
</tr>
<tr>
<td>GC</td>
<td>germinal center</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid-induced tumor necrosis factor ligand</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage–colony-stimulating factor</td>
</tr>
<tr>
<td>GP</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HPA</td>
<td>human platelet antigen</td>
</tr>
<tr>
<td>HPV</td>
<td>human herpes virus</td>
</tr>
<tr>
<td>IBLS</td>
<td>ITP Bleeding Scale</td>
</tr>
<tr>
<td>ICH</td>
<td>intracranial hemorrhage</td>
</tr>
<tr>
<td>IDO1</td>
<td>indoleamine 2,3-dioxygenase 1</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IPF</td>
<td>immature platelet fraction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>ITP</td>
<td>immune thrombocytopenia</td>
</tr>
<tr>
<td>ITP-BAT</td>
<td>ITP specific Bleeding Assessment Tool</td>
</tr>
<tr>
<td>IVIg</td>
<td>intravenous immunoglobulin</td>
</tr>
<tr>
<td>IWG</td>
<td>international working group</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
</tr>
<tr>
<td>mDC</td>
<td>migratory dendritic cell or myeloid dendritic cell</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMR</td>
<td>macrophage mannose receptor</td>
</tr>
<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>NAP-2</td>
<td>neutrophil-activating peptide-2</td>
</tr>
<tr>
<td>ND</td>
<td>non-depleted</td>
</tr>
<tr>
<td>NET</td>
<td>neutrophil extracellular trap</td>
</tr>
<tr>
<td>OCS</td>
<td>open canalicular system</td>
</tr>
<tr>
<td>PAR</td>
<td>protease-activated receptor</td>
</tr>
<tr>
<td>PAT</td>
<td>passive alloimmune thrombocytopenia</td>
</tr>
<tr>
<td>PBP</td>
<td>platelet basic protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PF-4</td>
<td>platelet factor-4</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>PTP</td>
<td>post-transfusion purpura</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoic acid-inducible gene-I</td>
</tr>
<tr>
<td>RLR</td>
<td>retinoic acid-inducible gene-I (RIG-I)-like receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SIGN</td>
<td>intercellular adhesion molecule-3-grabbing nonintegrin</td>
</tr>
<tr>
<td>SIRPα</td>
<td>signal regulatory protein α</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>T2-MZP</td>
<td>transitional 2 marginal-zone precursor</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T&lt;sub&gt;FH&lt;/sub&gt;</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TPO</td>
<td>thrombopoietin</td>
</tr>
<tr>
<td>TRAP</td>
<td>thrombin receptor activator peptide</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cells</td>
</tr>
<tr>
<td>TxA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>thromboxane A2</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WHO</td>
<td>the world health organization</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1 Preamble

This thesis focuses on the immune mechanisms mediating immune thrombocytopenia (ITP) and how therapies affect these responses.

ITP is defined as an isolated thrombocytopenia without other disorders that may cause thrombocytopenia. It is a consequence of increased platelet destruction in the periphery and/or impaired platelet production by megakaryocytes in the bone marrow. In Chapter One, I will first introduce a brief review on megakaryocyte and platelet biology and then provide an extensive review on ITP, including various immunological reasons that contribute to the platelet or megakaryocyte abnormalities in ITP. At the end of the introduction, I will introduce the aims of my study and hypotheses.

The original research work in the thesis will be introduced in Chapters Two to Four in a paper-based format. The work includes our studies on antibody- and T cell-mediated ITP and the effect of different treatments on the pathogenesis of the disease in two mouse models of ITP, i.e. the passive ITP and active ITP mouse models.

In Chapter Two, we examined antibody-mediated ITP in a passive mouse model with or without IVIg therapy. In particular, we were interested in how the anti-platelet antibodies affected the numbers and morphology of megakaryocytes in vivo without interference from T cell-mediated megakaryocyte destruction as this has not been well characterized. We also evaluated the effect of intravenous immunoglobulin (IVIg) therapy on platelet counts and the megakaryocytes in these mice.

Chapter Three focuses on T cell-mediated ITP without B cells, and the effectiveness of allogeneic platelet transfusions in alleviating the thrombocytopenia. While allogeneic platelet transfusions have been widely used clinically for decades in many other thrombocytopenic conditions, they are generally limited for use in patients with ITP. However, clinical evidence began to emerge that contradicted the “limited use” dogma showing the effectiveness of platelet
transfusions for the management of ITP. Moreover, whether there is any immunomodulatory effect of allogeneic platelet transfusions on the pathogenesis of ITP is unclear.

Next, we were interested in the interactions between B cells and T cells in ITP pathogenesis. One way to study this is by depleting B cells and monitoring the changes in T cell-mediated ITP. B cell depletion in vivo is a commonly used therapy for ITP and was initially thought to inhibit antibody-mediated ITP, however, it was found to have antibody-independent mechanisms as well. Therefore in Chapter Four, we examined the effect of B cell depletion therapy on T cell-mediated ITP in our active ITP mouse model.

Chapter Five will briefly summarize the key findings of the thesis, the limitations of the current studies and directions for future studies.
1.2 Megakaryocyte and Platelet Biology

1.2.1 Megakaryocyte development, differentiation, and platelet production

Mammalian megakaryocytes are a population of specialized bone marrow cells whose primary physiological role is to produce and release platelets into the peripheral circulation (Michelson, 2013). In both humans and mice, megakaryocytes initially develop from hematopoietic stem cells in the fetal yolk sac and shortly after, in the fetal liver and then later, mainly in the bone marrow after birth (Long et al., 1982; Machlus et al., 2014; Ogawa, 1993). Early megakaryocyte progenitors can be identified by surface markers such as CD34 and CD41, and transcription factors such as Flt3 (Battinelli et al., 2007). During their differentiation, they become lineage restricted and become promegakaryoblasts after they begin to express CD61 (also known as integrin β3 chain, or glycoprotein (GP)IIIa) and elevated CD41 (integrin αIIb chain, or GPIIb) molecules (Michelson, 2013).

Based on their morphological characteristics, megakaryocyte development can be divided into 3 different stages: megakaryoblasts, promegakaryocytes, and mature megakaryocytes (Dameshek and Miller, 1946; Long and Williams, 1981; Michelson, 2013). The megakaryoblasts are about 10-30μm in diameter with an irregular agranular nucleus (sometimes kidney-shaped) and a basophilic cytoplasm. The promegakaryocytes are approximately 15-80 μm in diameter with a more condensed nucleus, reduced nucleus/cytoplasm ratio and scant-to-moderate amounts of granules. The mature megakaryocytes on the other hand, may have a variable size from 18 to >100 μm in diameter with a condensed multilobed nucleus and a very low nucleus/cytoplasm ratio with plenty amounts of granules in the cytoplasm. Figure 1.1 highlights the major stages of megakaryocyte development (Ebbe, 1976; Machlus et al., 2014; Michelson, 2013).

There are several unique characteristics that occur during megakaryocyte development which are necessary for platelet production and eventual hemostasis. These include endomitosis and polyploidy, development of a demarcation membrane system (DMS), granule
In the bone, a common myeloid progenitor cell commits to megakaryocytic lineage, with the expression of lineage marker CD41 (integrin αIIb, GPIIb) on the cell surface. Another important lineage marker is CD61 (integrin β3, GPIIIa). The promegakaryoblast is the first morphologically recognizable megakaryocyte precursor in bone marrow, which then become a megakaryoblast, and eventually mature as a megakaryocyte. The megakaryocyte releases pro-platelets into circulation which then become platelets. Thrombopoietin (TPO) is the principle regulator during the megakaryocyte development.
formation/accumulation and proplatelet formation. The ploidy increase in megakaryocytes is thought to be essential for the significant gene transcription events required to make proteins for the approximately 1,000 platelets made from each megakaryocyte (Trowbridge et al., 1984). The mechanism of this process is not well understood but there are a few clues that have emerged and suggest both external growth factors and specific genetic processes are required. For example, it was found that endomitosis was critically dependent on a megakaryocyte growth factor, thrombopoietin (TPO), because defective signaling through its receptor, Mpl, leads to decreased megakaryocyte proliferation and increased circulating platelet volumes (Gurney et al., 1994). Actually, TPO is the most important growth factor during the whole process of megakaryocyte maturation, proplatelet formation and platelet production. Through binding to its receptor Mpl, TPO activates the tyrosine kinase Janus kinase 2 (JAK2) and triggers the activation of the JAK/STAT signaling pathway (STAT for ‘signal transducers and activators of transcription’) which further promotes megakaryocyte proliferation (Pallard et al., 1995; Sattler et al., 1995). In addition to TPO, IL-11 also stimulates the proliferation of megakaryocytes and ploidy maturation, synergistic to IL-3 (Kobayashi et al., 1993; Teramura et al., 1992). More recently, Kahr et al reported that mutations in both the human and mouse NBEAL2 gene induce decreased ploidy of megakaryocytes and developmental/structural defects such as a lack of alpha granules (Kahr et al., 2011; Kahr et al., 2013). Deppermann et al found similar alpha granule defects in Nbeal2−/− mice, but they did not observe megakaryocyte differentiation defects (Deppermann et al., 2013). In other studies, Mazharian et al showed that mutations in the SH2 domain-containing protein-tyrosine phosphatases, Shp1 and Shp2 were associated with arrested ploidy and decreased numbers of megakaryocytes together with a macrothrombocytopenia in vivo (Mazharian et al., 2013). These studies have contributed to our understanding of how megakaryocytes increase their ploidy.

The DMS system is an elaborate invaginated membrane system derived from the megakaryocyte plasma membrane and contacts both the extracellular environment and the megakaryocyte cytoplasm. It serves as a reservoir for extra membrane surface to generate proplatelets (Battinelli et al., 2007; Behnke, 1968, 1969; Nakao and Angrist, 1968; Radley and
Haller, 1982; Schulze et al., 2006). Schulze et al observed that megakaryocyte DMS invagination relies on actin polymerization (Schulze et al., 2006). A recent study by Eckly et al identified a pre-DMS structure and proposed DMS biogenesis in four steps: ‘(1) focal membrane assembly at the cell periphery; (2) plasma membrane invagination and formation of a perinuclear pre-DMS; (3) expansion through membrane delivery from Golgi complexes; and (4) ER-mediated lipid transfer.’ (Eckly et al., 2014)

During megakaryocyte maturation, another characteristic is the progressive formation and appearance of secretory granules, which are ultimately delivered to and stored in platelets. These granules are essential for platelet activation and function and there are several different types of granules in megakaryocyte and platelets, including α granules, dense granules, and lysosomes (Machlus et al., 2014). α-granules are the most abundant, usually 200-500nm in diameter containing hundreds of different proteins that are acquired through both protein synthesis and endocytosis/pinocytosis from the extracellular environment (Blair and Flaumenhaft, 2009; Handagama et al., 1987). Endogenously synthesized proteins include, for example, platelet factor 4, β-thromboglobulin, and von Willebrand factor (vWF). Exogenous proteins captured by the megakaryocytes and platelets include fibrinogen, albumin and factor V etc (Blair and Flaumenhaft, 2009; de Larouziere et al., 1998). Another type of secretory granules called dense granules are approximately 250 nm in diameter and contain, for example, serotonin, a non-metabolic pool of adenine nucleotides [adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP)], high concentrations of calcium, and other bioactive substances that are released upon platelet activation (Youssefian and Cramer, 2000).

During the final stages of megakaryocyte maturation, the cytoplasm begins to form proplatelets, which are released into the bloodstream and eventually become circulating platelets. The development of proplatelets from megakaryocytes has been visualized in vitro and in vivo (Behnke, 1969; Italiano et al., 1999; Junt et al., 2007; Patel et al., 2005). Proplatelet production begins with the extension of pseudopodia from the plasma membrane with subsequent elongation to yield thick proplatelet branches and eventually bifurcating the proplatelet ends to form thinner proplatelet shafts (Italiano et al., 1999). This process occurs
repetitively until the entire cytoplasm of megakaryocyte is consumed (Italiano et al., 1999; Patel et al., 2005). The proplatelet elongation is driven by microtubules at a speed of approximately 1μm/min and bifurcation is driven by actin polymerization (Hartwig and Italiano, 2006; Italiano et al., 1999; Tablin et al., 1990). Microtubule arrays extend throughout the entire membrane system and use a dynein-powered motor to slide bidirectionally to transport protein cargo from the megakaryocyte cytoplasm into the developing proplatelets (Hartwig and Italiano, 2006; Patel et al., 2005). The microtubules also function as scaffolds to move mitochondria and granules from the megakaryocytes to the developing proplatelets (Richardson et al., 2005). Furthermore, the extracellular matrix has also been shown to be involved in megakaryocyte maturation and proplatelet production. Matrix proteins such as vitronectin, collagen, and fibrinogen have all been shown to regulate proplatelet formation, possibly through interaction with integrins and adhesion receptors on the megakaryocytes surface which provide stimulatory signals for protein synthesis and skeletal remodeling (Balduini et al., 2008; Kunert et al., 2009; Larson and Watson, 2006; Sabri et al., 2004; Thon and Italiano, 2010). During proplatelet formation, megakaryocytes migrate from the bone marrow niches to the extravascular area and release proplatelets directly into the circulation (Junt et al., 2007). Ultimately, the released proplatelets divide and become platelets that are then available to maintain hemostasis (Figure 1.1) (Michelson, 2013).

1.2.2 Platelets in hemostasis and thrombosis

Platelets were first described independently by Gulliver and Addison and Donné in the 1840s. Subsequently, detailed drawings of platelets by Osler in the 1870s distinguished them from leukocytes and red blood cells (RBCs) (Robb-Smith, 1967). In 1882, Bizzozero observed platelets microscopically in the circulating blood of living animals and showed that they were the first component of the blood to adhere to damaged blood vessel walls (Bizzozero, 1881; Blanchette and Freedman, 1998). In 1910, James Wright coined the term ‘platelets’ which has now been universally accepted (Brown, 1913).

Platelets are anucleated cells with a biconvex discoid structure and are the smallest circulating cell type in the blood, with a size of approximately 2-4μm in diameter in humans and
1-3μm in mouse (Michelson, 2013; Thon and Italiano, 2010; Trowbridge et al., 1984). The normal platelet count in human peripheral blood is between 150 - 450×10⁹/L and their life span is approximately 10 days (Harker and Finch, 1969; Michelson, 2013; Nugent et al., 2009) whereas in mice, the normal platelet count (depending on the strain) is between 600 - 1600×10⁹/L with a life span of approximately 3-5 days (Michelson, 2013).

The primary physiological function of platelets is the maintenance of hemostasis. Upon vascular damage, platelets are rapidly activated and recruited to the site of injury and eventually form a platelet plug to stop bleeding and infectious microbe dissemination. Our understanding of the biological and molecular mechanisms has been largely improved in the last few decades.

Platelets have unique structures that are closely related to their hemostatic functions. For example, their membranes (like the DMS in megakaryocytes) are invaginated, providing additional membrane area during activation. These invaginations form the open canalicular system (OCS, or ‘surface-connected canalicular system’) which communicates with the extracellular environment and can allow extracellular proteins to be readily taken up by platelets (Michelson, 2013; White and Escolar, 1993). Platelets also have a dense tubular system which is the site of calcium storage and contain α and dense granules which are derived from megakaryocytes and can be secreted upon platelet activation. (Rodan and Feinstein, 1976). Probably the most important feature of platelets and their ability to mediate hemostasis is the expression of a variety of surface receptors including (1) integrin receptors αIIbβ3 (also known as GPIIbIIIa, or the CD41/CD61 complex) and α2β1 (GPIaIIa), (2) the leucine-rich repeat family receptor GPIb-IX-V, (3) the G protein-coupled receptors (GPCRs) including protease activation receptor 1 (PAR1) and PAR4 and the ADP receptors, P2Y₁ and P2Y₁₂, (4) immunoglobulin receptor GPVI, and (5) the tyrosine kinase receptor Mpl, etc. Upon activation, platelets also express additional receptors which further promote platelet function. For example, the C-type lectin receptor P-selectin is expressed on activated platelets and facilitates platelet adhesion.
Of these receptors, αIIbβ3 is unique to platelets and critical for platelet activation and aggregation. It is the most abundant glycoprotein on the platelet plasma membrane, with approximately 80,000 copies per platelet. αIIbβ3 can bind multiple ligands, such as fibrinogen, vWF, fibronectin, vitronectin etc. When platelets are activated, the αIIbβ3 transforms from a low affinity resting state into a high affinity state and this conformational change promotes its binding to its ligand, such as vWF and fibrinogen; a process known as ‘inside-out signaling’ (Plow and Ma, 2007).

Upon vascular injury, circulating platelets become exposed to the vascular subendothelial matrix, and undergo adhesion, activation, aggregation and eventually form a plug that physically stops the flow of blood out of the vessel. Initially, vascular injury exposes collagen which can bind to platelet receptors GPIaIIa and GPVI as well as vWF and the immobilized vWF is recognized by platelet GPIb-IX-V, initiating platelet adhesion. Platelet adhesion is followed by platelet activation which is characterized by a series of changes in platelets including thromboxane A2 (TxA2) synthesis, activation of αIIbβ3 (inside-out signaling), platelet cytosol calcium elevation, increased surface expression of multiple receptors as well as platelet shape change (platelet spreading) and granule secretion. The activated αIIbβ3 molecules can bind to vWF and fibrinogen with high affinity and this can then bridge activated αIIbβ3 receptors on different platelets resulting in platelet aggregation. In addition, elevated calcium elevation also induces platelet shape change and phosphatidylserine (PS) exposure on platelet surface which provides a procoagulant surface and promotes thrombin generation. Concurrently, granule secretion releases bioactive substances including vWF, fibrinogen, ADP and these released soluble platelet agonists further amplify platelet activation and aggregation through interaction with their receptors e.g. between ADP and P2Y1 and P2Y12 receptors, thrombin and PAR1 and PAR4, TxA2 and GPCR (Furie and Furie, 2008; Li et al., 2010).

Platelet activation and aggregation is also amplified by the activation of the coagulation cascade. This cascade is activated when tissue factor molecules are exposed on injured endothelial or subendothelial cells and when exposed to the circulation, it leads to the production of thrombin and the formation of fibrin. Thrombin could further promote αIIbβ3
activation and platelet aggregation. Fibrin polymerizes into long threads and forms a network that further traps platelets. Eventually with the accumulation of platelet aggregation and fibrin deposition, the formed platelet plug is stabilized which arrests blood loss from the injured site and this can subsequently recruit leukocytes for healing (Furie and Furie, 2008; Li et al., 2010).

1.2.3 The immunomodulatory role of platelets

Although hemostasis is the primary role for platelets, it has become clear that they are also immune-like cells with innate immune functions and with the capability to interact with adaptive immune cells. Platelets are involved in many infectious diseases and have the ability to recognize and bind to viruses, bacteria, and parasites (Assinger, 2014; Kapur et al., 2015b; Yeaman, 2010). To list just a few of the organisms that are able to directly interact with platelets are the human immunodeficiency virus (HIV), hepatitis C virus (HCV), Epstein–Barr virus (EBV), *Staphylococcus aureus*, *Streptococcus sanguis* and malarial parasites (Assinger, 2014; Yeaman, 2010).

Platelets can directly recognize pathogens through their extensive repertoire of surface receptors. For example, Hantavirus and adenoviruses as well as bacteria *S. aureus* have been shown interact with platelets via GPIIbIIIa (Cheung et al., 1991; Mackow and Gavrilovskaya, 2001; Niemann et al., 2004; Yeaman, 2010). Platelets can also interact with some viruses and bacteria through their GPIaIIa and GPVI receptors (Assinger, 2014; Coulson et al., 1997; Flaujac et al., 2010; Zahn et al., 2006). Besides their various receptors that are important in hemostasis, platelets also express toll-like receptors (TLRs) and lectin receptors that are specialized for infectious antigen detection. TLRs are a family of proteins that have protective properties against microbial infections by recognizing structurally conserved molecules of microbes and activating the host immune system (McKenzie et al., 2013). It has been shown that human platelets express all TLRs 1-9 (Kapur et al., 2015b) but whether they express TLR10 has not been reported. Among those receptors, TLR4 is the most studied and has been shown to be functional on platelets (Andonegui et al., 2005; Aslam et al., 2006). The activation of platelets through TLR4 is triggered by the binding of TLR4 to lipopolysaccharides (LPS), a core
antigen in the outer membrane of Gram-negative bacteria (Andonegui et al., 2005). This process can further promote platelet-neutrophil interaction and the subsequent formation of neutrophil extracellular traps (NETs) (Andonegui et al., 2005; Clark et al., 2007). Lectin receptors such as the C-type lectin domain family 2 (CLEC-2) on platelets have also been shown to be able to bind HIV and dengue virus (Assinger, 2014; Chaipan et al., 2006). Moreover, platelets can detect infectious agents through their complement (C1q) receptor and chemokine receptors (CCR) CCR1, CCR3 and CCR4 etc (Assinger, 2014; Clemetson et al., 2000; Nguyen et al., 2000; Othman et al., 2007; Scott et al., 1987; Yeaman, 2010). In addition to direct antigen binding, human platelets also express FcγRII and FcεRI receptors through which they can detect antigen-immunoglobulin complexes (Joseph et al., 1983; Rosenfeld et al., 1985; Yeaman, 2010).

All the receptors mentioned above could induce platelet activation and promote a series of responses as will be introduced below. Perhaps the most common pathway of platelet activation upon exposure of exogenous infections is through thrombin production and this is because a broad range of microbial agents can trigger the release of tissue factor from injured endothelial cells and subendothelial stroma. Nonetheless, the mechanisms of how platelet activation in vivo is induced after exposure to infectious agents is probably multifactorial (Smyth et al., 2009).

Upon activation, platelets can kill or help to kill pathogens in many ways. Firstly, through degranulation, platelets can release a variety of microbialcidal proteins such as kinocidins, platelet factor-4 (PF4/ CXCL4), β-defensins, all of which have anti-microbial characteristics (Assinger, 2014; Auerbach et al., 2012; Mohan et al., 2010; Solomon Tsegaye et al., 2013; Wilson et al., 2013; Yeaman, 2010). Secondly, through the upregulation of CD40L (CD154) expression on platelets, the interaction between platelets and CD40 expressing endothelial cells promotes several inflammatory reactions (Henn et al., 1998). Thirdly, activated platelets express P-selectin which interacts with its counter-receptor P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of leukocytes (Polgar et al., 2005). Fourthly, activated platelets, via chemokine secretion, can recruit dendritic cells (DCs) and monocytes and promote their maturation (Hagihara et al., 2004; Langer et al., 2007; Metcalf Pate et al., 2013; Singh et
al., 2012). Fifthly, platelets can interact with neutrophils which not only promotes neutrophil extracellular trap (NET) formation for trapping pathogens but also stimulates reactive oxygen species (ROS) production and boosts phagocytosis by neutrophils (Elzey et al., 2005; Henn et al., 1998; Yeaman, 2010).

Platelets also have other active immunomodulatory roles on the immune system. For example, we previously showed improved skin graft survival after allogeneic platelet transfusions (Aslam et al., 2008). In addition, part of my PhD work has investigated the immunomodulatory role of allogeneic platelet transfusions in a murine model of immune thrombocytopenia (ITP) (Guo et al., 2014).

### 1.2.4 Thrombocytopenia induced by immune responses against platelets

Platelets can be the target of pathological immune responses which lead to increased platelet destruction and/or decreased platelet production. This eventually causes the development of thrombocytopenia, which is usually defined as the peripheral platelet count below $150 \times 10^9/L$ in humans (Greer, 2014). Thrombocytopenia mediated by immunological factors can be generally divided into two categories: autoimmune and alloimmune thrombocytopenia. Thrombocytopenia caused by autoimmune mechanisms is induced by abnormal immune responses against self-platelet antigens, including primary immune thrombocytopenia (ITP) or drug/infection-induced secondary ITP. In contrast, alloimmune thrombocytopenia refers to thrombocytopenia induced after allogeneic platelet transfusions where antibodies develop against the transfused platelet alloantigens (Greer, 2014). Platelet-specific alloimmune responses can also induce thrombocytopenia and are exclusively triggered by allelic human platelet antigens (HPA) (Hayashi and Hirayama, 2015). The differences in HPA antigens can induce alloimmune responses that cause fetal and neonatal alloimmune thrombocytopenia (FNAIT), post-transfusion thrombocytopenia (PTP), and passive transfer of platelet alloantibodies.

#### 1.2.4.1 Fetal and neonatal alloimmune thrombocytopenia (FNAIT)
Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is also known as neonatal alloimmune thrombocytopenia (NAIT or NAITP or NATP). It is characterized by thrombocytopenia in the fetus or newborn and develops when the mother is negative for a certain HPA antigen but carries a fetus who inherits the HPA antigen from the father. Subsequently, the HPA on fetal platelets can be recognized as foreign by the mother’s immune system and the mother develops IgG anti-HPA antibodies that migrate across the placenta, bind to the fetal platelets and/or megakaryocytes and induce fetal thrombocytopenia (Kamphuis et al., 2014; Liu et al., 2015). The most common HPA epitopes on platelets are HPA-1a located on GPIIIa, and HPA-5b on GPIa (Mueller-Eckhardt et al., 1989; Thienelt and Calverley, 2009; von dem Borne et al., 1981). In Caucasians, 85% of FNAIT is induced by anti-HPA-1a alloantibodies (Kamphuis et al., 2010) but although the mismatch of HPA between the mother and the fetus is as high as 1/50, the incidence of severe FNAIT (platelet count <50x10^9/L) is below 1/500 (Kamphuis et al., 2014; Thienelt and Calverley, 2009). FNAIT may develop during the first pregnancy with a recurrence rate of 80% during subsequent pregnancies (Radder et al., 2003). It is usually diagnosed after the presence of bleeding symptoms such as petechiae or after detection of intracranial hemorrhage (ICH) by ultrasound examination in the newborns. ICH may be life-threatening and happens in 10-20% of severe FNAIT cases (Kamphuis et al., 2014). Intravenous immunoglobulin (IVIg) and platelet transfusions are the recommended treatment for newborns with severe thrombocytopenia (Murphy and Bussel, 2007). Due to the high recurrence rate, prophylactic antenatal treatment is provided for pregnant women with a history of FNAIT, especially for mothers with previous pregnancies associated with ICH. High dose (0.5-2g/Kg) IVIg combined with steroids is the most common treatment.

1.2.4.2 Post-transfusion purpura (PTP)

Post-transfusion purpura (PTP) is a rare adverse complication after red blood cell or platelet transfusions which is mediated by alloantibodies against HPA antigens. It usually abruptly develops 7-10 days after the transfusion and symptoms are very similar to those seen in patients with ITP (Chapman et al., 1987; Shulman et al., 1961; Thienelt and Calverley, 2009; Vogelsang et al., 1986). Patients may have widespread purpura and in some severe cases, life-threatening
bleeding symptoms such as ICH (Li et al., 2012a). PTP occurs most commonly in women who have had history of multiple pregnancies or transfusions. It appears that patients who have developed alloimmune responses against HPAs and re-exposed to the same antigens after transfusion produce high titers anti-HPA antibodies. The most common antigens involved in PTP are HPA-1a, HPA-1b, HPA-3a, HPA-3b, and HPA-5a (Hayashi and Hirayama, 2015; Kickler et al., 1988; Simon et al., 1988). The pathophysiology of PTP is unclear but several hypotheses have been proposed including (a) autologous platelets being destroyed because of binding of immune complexes to their surface; (b) recipient platelets acquiring the phenotype of the donor's platelets because they bind soluble antigens from the transfused blood product or (c) exposure to foreign transfused platelets induces the formation of cross-reactive autoantibodies against the recipient's platelets (Thienelt and Calverley, 2009). Although most cases of PTP are self-limiting, therapeutic interventions may shorten the period of thrombocytopenia and include IVIg, plasmapheresis, or steroids (Thienelt and Calverley, 2009).

1.2.4.3 Passive alloimmune thrombocytopenia (PAT)

Passive alloimmune thrombocytopenia (PAT) usually occurs within a few hours after a blood transfusion and is mediated by alloantibodies contained in the plasma of blood donors including alloantibodies against HPA-1a and HPA-5b (Santoso and Kiefel, 2001; Warkentin and Smith, 1997; Warkentin et al., 1992; Webert et al., 2014). Patients usually recover after the alloantibodies are cleared.
1.3 Immune thrombocytopenia (ITP)

Immune thrombocytopenia (ITP) is an autoimmune disorder and can be classified as either primary or secondary ITP. As primary ITP is the main focus of this thesis, from now on the abbreviation ITP will stand for primary chronic immune thrombocytopenia unless specified.

1.3.1 Brief history of ITP

The history of ITP has been investigated and comprehensively reviewed (Blanchette and Freedman, 1998; Freedman and Blanchette, 1998). The acronym “ITP” was historically defined as “idiopathic thrombocytopenic purpura” but the disorder has had several terms associated with it including “autoimmune thrombocytopenic purpura”, “immune thrombocytopenic purpura” and “autoimmune thrombocytopenia”. Recently, because of the improved understanding of the immunological pathogenesis and diagnosis of ITP, the disease’s nomenclature has been standardized and is now termed “immune thrombocytopenia” (Rodeghiero et al., 2009).

The word “purpura” was derived from the Greek word porphyra (Πρόφυρα) which is a name of a purple fish from which a purple dye was obtained (Freedman and Blanchette, 1998). Purpura has been used in medicine to describe a clinical symptom characterized with “red spots” or “red eminences” during, for example, pestilential fevers in the Greco-Roman period and was coined by the ancient physicians Hippocrates and Galen. In the 10th century, purpura in its chronic form was described by an Arab physician Avicenna (Blanchette and Freedman, 1998; Jones and Tocantins, 1933). In 1580, purpura without fever was described by Amatus Lusitanus in his “Curatonum Medicinalium centuriae septem” (Blanchette and Freedman, 1998; Lusitanus, 1965) and in 1658, Lazarus de la Rivière (Riverius), physician to the King of France, described purpura as “purple spots like Flea-bitings, called...Peliculae or Pelechiae ... ” (Blanchette and Freedman, 1998; Riverius, 1668).

Paul Gottleib Werlhof is credited with probably the first description of ITP in 1735 (Morbus Maculosus Haemorrhagicus). Subsequently, ITP was also known as Werlhof’s Disease. “In his Opera Omnia (printed posthumously in 1775) he described `an adult girl,
robust, without manifest cause,' who bled from her nose and mouth and vomited `very thick, extremely black blood. Immediately there appeared about the neck & on the arms, spots partly black, partly violaceous or purple...'.” (Blanchette and Freedman, 1998; Werlhof, 1965). In 1802, Robert Willan, a London physician distinguished four types of purpura in his book “On Cutaneous Diseases” (Blanchette and Freedman, 1998; Willan, 1808).

Although platelets were discovered in the 1840s by Gulliver, Addison and Donné, the relationship between platelets and purpura was not discovered until 1883 by Krauss in Germany and 1887 by Denys in Louvain (Blanchette and Freedman, 1998; Denys, 1887; Krauss, 1883; Robb-Smith, 1967). In 1889, Georges Hayem in Paris documented the first actual platelet count in a patient with purpura (Blanchette and Freedman, 1998; Denys, 1887; Krauss, 1883). In 1915, Frank in Poland described normal numbers of megakaryocytes in ITP (Blanchette and Freedman, 1998; Frank, 1915) and a year later, George Minot in the USA studied a case of ITP in detail (Blanchette and Freedman, 1998; Minot, 1916). In the same year, Kaznelson in Prague described the first case of splenectomy as a successful therapy in a patient with ITP and since then, splenectomy has become an important treatment (Blanchette and Freedman, 1998; Kaznelson, 1916). The success of splenectomy in ITP treatment also provided solid evidence to support the theory that the development of ITP is due to platelet destruction in the spleen. During that time, however, an alternative theory supported by Frank et al was that ITP was related to decreased platelet production (Blanchette and Freedman, 1998). Now it has been recognized that both theories are correct in patients with ITP.

In the second half of the twentieth century, the understanding of the pathogenesis of ITP as well as the therapeutic management of the disease was greatly improved. For example, 1951 was an important year in the history of ITP. Harrington et al showed for the first time that a factor in the plasma of patients with ITP was responsible for inducing thrombocytopenia; transferring plasma from patients with ITP into healthy volunteers including himself induced significant thrombocytopenia in most recipients (Harrington et al., 1951). This classic experiment provided substantial evidence that there is a factor in the plasma that could directly induce thrombocytopenia. In the same year, Hirsch and Dameshek differentiated the acute and

| Primary ITP | **Primary ITP** is an autoimmune disorder characterized by isolated thrombocytopenia (peripheral blood platelet count $<100 \times 10^9/L$) in the absence of other causes or disorders that may be associated with thrombocytopenia.  

**The diagnosis of primary ITP** remains one of exclusion; no robust clinical or laboratory parameters are currently available to establish its diagnosis with accuracy.  

**The main clinical problem of primary ITP** is an increased risk of bleeding, although bleeding symptoms may not always be present. |
|---|---|
| Secondary ITP | All forms of immune-mediated  

Thrombocytopenia except primary ITP |
| Phases of the disease |  

- **Newly diagnosed ITP:** within 3 months from diagnosis  

- **Persistent ITP:** between 3 to 12 months from diagnosis.  

Includes patients not reaching spontaneous remission or not maintaining complete response off therapy. |
- **Chronic ITP**: lasting for more than 12 months
- **Severe ITP**: Presence of bleeding symptoms at presentation sufficient to mandate treatment, or occurrence of new bleeding symptoms requiring additional therapeutic intervention with a different platelet-enhancing agent or an increased dose.
chronic forms of ITP (Hirsch and Dameshek, 1951). Moreover, the first application of corticosteroids for the treatment of ITP was also performed by Wintrobe et al and corticosteroids have now become the major first-line therapy for ITP (Blanchette and Freedman, 1998; Wintrobe et al., 1951). In 1965, Shulman et al identified the plasma factor belonging to the IgG fraction of plasma and in the mid 1970’s, Dixon and Cines demonstrated that platelet-associated IgG could be quantified and verified that the plasma factor was actually anti-platelet antibodies (Blanchette and Freedman, 1998; Cines and Schreiber, 1979; Dixon et al., 1975; Shulman et al., 1965). In 1981, Imbach reported the first application of IVIg for the treatment of ITP (Imbach et al., 1981) and in 1991 Semple et al found increased platelet-reactive IL-2 secreting CD4+ T helper (Th) cells in patients with ITP which provided probably the first evidence of abnormal T cell reactivity in the development of ITP (Semple and Freedman, 1991). It has now been widely accepted that the etiology of ITP includes both humoral and cellular immune responses and in the last two decades, a considerable amount of immune investigations have been published on ITP.

1.3.2 Current definition and diagnosis of ITP

It is now well understood that ITP is an autoimmune disease of children and adults, characterized with an insufficient number of platelets in the circulation due to decreased platelet production in the bone marrow and/or increased peripheral platelet destruction in the spleen. According to the International Working Group (IWG) report, primary ITP is now defined as “an autoimmune disorder characterized by an isolated thrombocytopenia (peripheral blood platelet count <100 × 10^9/L) in the absence of other causes or disorders that may be associated with thrombocytopenia. The diagnosis of primary ITP remains one of exclusion; no robust clinical or laboratory parameters are currently available to establish its diagnosis with accuracy. The main clinical problem of primary ITP is an increased risk of bleeding, although bleeding symptoms may not always be present” (Rodeghiero et al., 2009). Secondary ITP refers to immune-related thrombocytopenia secondary to other etiologies, such as those caused by drugs,
infections or other diseases e.g. Lupus. The proposed definition of ITP as well as phases of ITP are summarized in Table 1.1.

In the IWG report, the phases of ITP have also been significantly altered and are no longer simply divided into acute or chronic, but instead are now divided into 3 categories: newly diagnosed (from diagnosis to 3 months), persistent (3-12 months after diagnosis) and chronic (more than 12 months after diagnosis)(Rodeghiero et al., 2009). An advantage of the new diagnostic criteria is avoiding the vagueness of ITP at diagnosis, at which time point patients may have developed the disease recently or alternatively may have had the disease asymptptomatically for a long time. Persistent ITP includes patients who did not achieve spontaneous remission or no response after initial treatment but within a year from initial diagnosis, they were able to eventually maintain a response after treatment. Patients who remain thrombocytopenic after 12 months from diagnosis are categorized as chronic ITP. Patients with newly diagnosed and persistent ITP have significantly higher remission rates than patients with the chronic form of ITP (Imbach et al., 2006; Rodeghiero et al., 2009; Sailer et al., 2006; Stasi et al., 1995). Therefore more aggressive treatment was recommended for patients with the chronic form of the disorder (Rodeghiero et al., 2009).

**Incidence and prevalence of ITP**

The incidence of ITP varies according to age, gender and geographical areas. The incidence of childhood ITP is approximately 4-5/100,000 person-year in Europe and 8/100,000 in North America, with a peak incidence at the age of 1-5 years (median 4 years) with a male dominated pattern (Hedman et al., 1997; Kuhne et al., 2001; Segal and Powe, 2006; Terrell et al., 2012; Yong et al., 2010; Zeller et al., 2005). The prevalence of ITP in children is about 4.6/100,000 children in Europe and 7.2/100,000 in North America (Hedman et al., 1997; Segal and Powe, 2006). The incidence of childhood ITP is also influenced by the season and prevalence of infections, showing a peak of new diagnoses in winter, especially in February, and platelet count nadirs in autumn (Kuhne et al., 2001; Moulis et al., 2014; Zeller et al., 2005). In adults, the overall incidence of ITP is about 2.9/100,000 in European countries and about
Figure 1.2: Petechiae in the skin of patients with ITP. Adopted from Greer, J.P. (2014), Wintrobe's clinical hematology (Philadelphia: Wolters Kluwer, Lippincott Williams & Wilkins Health), Figures 45.1 and 47.4. (Greer, 2014).
10-12/100,000 person-year in North America (Koylu et al., 2015; Moulis et al., 2014; Segal and Powe, 2006; Terrell et al., 2012). The prevalence of adult ITP is approximately 35-50/100,000 in Europe and about 9-10/100,000 in North America but the latter prevalence may be underestimated due to the limitation of local registries (Koylu et al., 2015; Moulis et al., 2014; Segal and Powe, 2006; Terrell et al., 2012). In adult ITP, the incidence is higher in women than men in patients below age 64, and vice versa in patients of age 65 and older (Bennett et al., 2011; Terrell et al., 2012). The overall incidence and prevalence of ITP is also increased after 65, at about 90/100,000 in the UK and 20/100,000 in North America (Bennett et al., 2011; Terrell et al., 2012). A limitation to these statistics is the lack of consistency of clinical criteria among the reported investigations. Although the standard for the definition and diagnostic criteria has been recommended by the IWG in 2009, the published data that adhere to the IWG are still limited (Rodeghiero et al., 2009). In children, the onset of ITP is usually preceded by an infectious history within 3 weeks and infections such as Varicella zoster virus or Epstein-Barr virus infection tend to be the most common (Kuhne et al., 2003; Liel et al., 2014; Lusher and Iyer, 1977; Lusher and Zuelzer, 1966; Provan et al., 2010). In addition, Measles-Mumps-Rubella (MMR) vaccination may also lead to an increased risk of ITP (Miller et al., 2001; Provan et al., 2010). In adults, the onset of ITP is usually insidious and an infectious history is less common (den Ottolander et al., 1984; Jacobs et al., 1986; Liel et al., 2014; Pizzuto and Ambriz, 1984; Stasi et al., 1995).

**Clinical manifestations of ITP**

Clinical manifestations of ITP are mainly hemorrhagic symptoms such as purpura, bruising in the skin, oral cavity bleeding, gastrointestinal (GI) bleeding and/or rarely, intracranial hemorrhage (ICH). The definition and category of bleeding manifestations was also provided by the IWG recently and is listed in Appendix I (Rodeghiero et al., 2013). As mentioned in Appendix I, petechiae are a typical symptom of ITP and refer to small dots in the skin caused by intradermal hemorrhages. They are red when fresh and become purplish within a few days as shown in Figure 1.2, adopted from Greer, J.P. (2014), Wintrobe’s clinical hematology (Greer, 2014). Ecchymoses (bruises) are another type of typical bleeding symptom
in the skin, larger than petechiae and are usually observed on the back and thighs. Other bleeding symptoms include hemATOMA (localized swelling filled with blood), epistaxis (nasal bleeding), menorrhagia (excessive amount of menstrual bleeding) hematochezia (stools with blood) and ICH, etc.

In general the severity of bleeding correlates with the platelet counts (Bolton-Maggs and Moon, 1997; Cines et al., 2009; Kuhne et al., 2001; Neunert et al., 2008; Page et al., 2007) but in patients with platelet counts less than 20×10^9/L, there is no significant correlation. It has been controversial whether the management of ITP should be based on absolute platelet count or bleeding symptoms. Therefore, investigation and validation of bleeding assessment in ITP would provide a useful guidance for the management of ITP.

With these considerations, efforts have been made since the 1990s to produce different bleeding assessment tools (Bolton-Maggs et al., 2004; Bolton-Maggs and Moon, 1997; Freedman, 2003). For example, in 1997, Bolton-Maggs categorized pediatric ITP patients registered in the UK into four groups: “no symptoms; mild symptoms (bruising and petechiae, occasional minor epistaxis, very little or no interference with daily living); moderate symptoms (more severe skin manifestations with some mucosal lesions, and more troublesome epistaxis and menorrhagia); and severe symptoms (bleeding episodes [epistaxis, melaena, and/or menorrhagia] requiring hospital admission and/or blood transfusion— symptoms interfering seriously with quality of life).” (Bolton-Maggs and Moon, 1997). Although these four categories seem to correlate with platelet counts ranging from <10, 10-19, 20-50, 51-100 to >100 (×10^9/L), most patients only had mild symptoms in all groups. Therefore this assessment tool was not specific enough to indicate the severity of the disease or provide any prognostic clues and this was confirmed by similar findings in the following decade (Kuhne et al., 2001; Neunert et al., 2008). In 2002, Buchanan and Adix proposed another bleeding assessment criteria that includes overall bleeding assessment with 6 grades from none to life-threatening as well as 5 grades of epistaxis, oral bleeding and skin bleeding (Buchanan and Adix, 2002). However there was a lack of assessment of the prognostic value in their studies. The world health organization (WHO) also provided an evaluation tool for hemorrhage/bleeding in 2006, which was designed for
adverse event evaluation after chemotherapy in cancer patients (NCI [2006] Common Terminology Criteria for Adverse Events [CTCAE] version 3.0. National Cancer Institute, U.S. National Institutes of Health. Available at http://ctep.info.nih.gov.). This tool provided grad of 1 to 5 for hemorrhage, based not only on the manifestation but also on the medical intervention and outcome; for example 5 refer to death. In 2007, Page et al proposed an advanced assessment tool, the “*ITP Bleeding Scale (IBLS)*” (Page et al., 2007). In the IBLS, bleeding symptoms were assessed at nine anatomical sites including the skin, oral, nasal, GI, urinary, gynecological, pulmonary, intracranial and subconjuntival sites. Bleeding in each individual site could be further graded from 0 to 2 based on the severity. Similar to Bolton-Maggs Assessment tool, although the bleeding grade correlated with platelet counts in all patients, the IBLS score did not correlate when platelet counts in patients were below 30×10⁹/L (Bolton-Maggs and Moon, 1997; Page et al., 2007). Despite this, the IBLS provided a useful tool to capture more details in bleeding manifestations as compared to previous assessment tools. More recently, the IWG provided an ITP specific Bleeding Assessment Tool (ITP-BAT), which provided a systemic evaluation including “*skin (S), visible mucosae (M), and organs (O), with gradation of severity (SMOG)*” with clear definitions of the manifestations and descriptions of grading standards (listed in Appendix I) (Rodeghiero et al., 2013). Under each of the “*S*” “*M*” and “*G*” categories, the bleeding severity could be further graded from 0 to 4 and the highest grade would be taken if there are more than one symptom within in the category (Rodeghiero et al., 2013). In this way, the ITP-BAT not only allows physicians to distinguish bleeding in different sites to allow more consistent evaluations but also aids in quicker decision making for ITP management. Although ITP-BAT provides the most refined bleeding assessment standard to date, its application needs to be validated and the relationship between the severity of bleeding symptoms and the outcome of the patients with/without treatment need to be further investigated (Rodeghiero et al., 2013). Despite the lack of standardization in bleeding assessments, it has been shown that ITP patients with a bleeding history have a significantly higher risk of bleeding than those without (Rodeghiero et al., 2013). In pediatric ITP, more than half of the patients would have spontaneous remission without any intervention, although most patients only have mild skin
manifestations, whereas ICH is rare but has a mortality of 25% (Kuhne et al., 2001; Psaila et al., 2009; Zeller et al., 2005).

Efforts have been made to look for prognostic markers to predict the bleeding risk in ITP. Recently, there are a few studies on the immature platelet fraction (IPF) and function of platelets in ITP. IPF is a technical description of a circulating platelet population characterized by a larger flow cytometric forward light scatter which, in general, indicates a larger diameter of the platelets (Briggs et al., 2004). In 2011, two groups independently reported increased IPF in the peripheral blood of patients with ITP (Barsam et al., 2011; Psaila et al., 2009). The increase in IPF percentage in ITP, as compared to control healthy volunteers, indicates more platelet production and more immature platelets in the circulation and was found associated with less clinically severe bleeding events (Barsam et al., 2011). Moreover, IPF was found significantly increased in pediatric patients with chronic ITP compared with patients with acute ITP, which may thus be an useful prognostic tool (Adly et al., 2015). In 2014, van Bladel et al found an inverse correlation between bleeding events and platelet functions in pediatric chronic ITP (van Bladel et al., 2014). They first used a microaggregation test to test the aggregation potential of isolated platelets by flow cytometry (van Bladel et al., 2014). In addition, platelets were stimulated with adenosine diphosphate (ADP), convulxin (CVX), thrombin receptor activator peptide (TRAP), GPVI and proteinase-activated receptor 1 receptors respectively and their activation level was measured using fluorescent-conjugated antibodies against P-selectin and fibrinogen (van Bladel et al., 2014). Frelinger et al have recently confirmed that platelet function, as indicated by TRAP-stimulated P-selectin and activated GPIIbIIIa and CD42b expression levels, is correlated with the severity of bleeding in pediatric ITP (Frelinger et al., 2015). Therefore, it appears that in the near future, prognostic markers/assays may be available for the prediction of bleeding events and for the improvement of ITP management.

**Laboratory tests for ITP**

Current laboratory findings are still non-specific in ITP. Blood tests most often reveal an isolated thrombocytopenia and sometimes show normocytic anemia proportional to blood loss.
White blood cell counts and differential counts are usually normal (Liel et al., 2014) and although more than half of the patients with ITP have detectable antiplatelet antibodies in their circulation, antiplatelet antibody tests are not commonly used clinically (Liel et al., 2014). This is generally because there are several limitations to the antibody testing. First, the reliability of the tests needs to be improved, as antiplatelet antibodies are found in some healthy individuals (Chong and Ho, 2005; Liel et al., 2014). The presence of antiplatelet antibodies in healthy individuals has also evoked further questions relating to the specificity of anti-platelet antibodies and the value of the test itself (Arnold et al., 2015). In addition, there is lack of evidence for the prognostic value of antiplatelet antibody tests and the tests are not readily available in most hospitals and therefore their application for clinical diagnosis is limited. Bone marrow examinations in ITP often reveal normal or an increased number of megakaryocytes, with an increased proportion of immature megakaryocytes (Dameshek and Miller, 1946; Houwerzijl et al., 2004).

**ITP is a diagnosis of exclusion**

As none of the symptoms or laboratory tests described above are specific enough and these characteristics are often observed in secondary ITP and other diseases, the diagnosis of ITP still remains one of exclusion (Provan et al., 2010). However, the history, physical examination, complete blood count and peripheral blood film need to be considered for diagnosis.

### 1.3.3 Pathogenesis of ITP

ITP is an autoimmune disease initiated by a breakdown of immunological self-tolerance (Cines et al., 2009). The consequence of the failure of self-tolerance results in decreased platelet production from megakaryocytes in the bone marrow and increased peripheral platelet destruction in the spleen (Cines et al., 2014; McKenzie et al., 2013). Although the development of anti-platelet specific immune responses in ITP is a complicated process that has not been completely understood, it has been shown to be related to but not limited to: potential platelet and megakaryocyte defects, contributions from antigen presenting cells (APCs), increases in autoreactive B and T cells, inefficient clearance of those autoreactive
Figure 1.3: A schematic view of the pathogenesis of ITP.
The intrinsic defects in megakaryocytes and platelets may contribute to the initial activation of the immune system. Other unknown environmental factors or microbial infections may also activate the megakaryocytes and/or platelets which then activate antigen presenting cells (APCs) to present platelet antigens and to stimulate autoreactive B and T cell responses. The activated B cells mature into plasma B cells and produce anti-platelet autoantibodies that could mediate platelet and megakaryocyte destruction with the help of Th1, Th17 and Th22 cells. These CD4\(^+\) T cells also promote the rise of platelet autoreactive CD8\(^+\) T cells (CTLs) which can induce direct platelet and megakaryocyte lysis. Defects in Bregs and Tregs as well as imbalances in cytokine production all contribute to the development of ITP.
immune cells, and dysregulation of cytokine production. A schematic view of the pathogenesis of ITP is shown in Figure 1.3.

1.3.3.1 Overview of self-tolerance

Under physiological situations, the immune system protects our body from exogenous infectious invasions, however does not attack self-antigens. This self-tolerance is maintained both in the thymus and bone marrow during T and B cell development (usually referred to as ‘central tolerance’), as well as in the periphery (peripheral tolerance) (Paul, 2013).

Thymic tolerance of T cells

T cells are characterized by the expression of T cell receptors (TCRs) on their cell surface which enables them to recognize virtually any peptide antigen presented by MHC molecules (Chaplin, 2010). TCRs are in association with CD3 (altogether form the TCR complex) and need support from their co-receptors: CD4 or CD8 (Huppa and Davis, 2013). The TCR complexes together with CD4 recognize peptide: MHC class II complexes whereas TCR together with CD8 recognize peptide: MHC class I complexes (Chaplin, 2010; Matsui et al., 1986).

T cells originate from hematopoietic stem cells in the bone marrow and migrate into the thymus for maturation (hence called “T” cells) (Boehm, 2008; Rothenberg and Champhekar, 2013). A key feature of T cells is the diversity of TCRs. TCRs genes are composed of multiple V, D and J gene segments. In the thymus, these early pre-T cells undergo VDJ rearrangement in their TCR beta genes leading to the functional expression of a rearranged β chain of the TCR on their cell surface. Cells that fail to generate functional β chains cannot proliferate (β-selection) and are eliminated whereas those T cells that successfully rearrange their β chains undergo extensive proliferation, express both CD4 and CD8 molecules (called double positive T cells) and eventually express a rearranged TCR-α chain (Paul, 2013). After this, T cells express either CD4 or CD8 exclusively (the CD4/CD8 lineage commitment) and eventually a small population of the cells mature from the thymus as either CD4⁺ or CD8⁺ naïve T cells (Shah and Zuniga-Pflucker, 2014).
During the process of T cell development, the most important two mechanisms of self-tolerance for T cells called ‘positive selection’ and ‘negative selection’ take place (Goodnow and Ohashi, 2013; Shah and Zuniga-Pflucker, 2014). Positive selection happens in double positive T cells that survived β-selection. It is a process in which only T cells that recognize self-peptide: MHC complexes expressed on thymic epithelial cells (TECs) receive survival signals from the TECs and proceed to further maturation. Those T cells that fail to recognize self MHC undergo apoptosis (self MHC restriction). Positive selection ensures the TCR can recognize either self MHC class I or class II molecules and enables the T cells distinguish self from non-self. Another mechanism termed negative selection happens after CD4/CD8 commitment. It is a process of deleting autoreactive T cells with high affinities of TCR for self-MHC (Laky and Fowlkes, 2007; Shah and Zuniga-Pflucker, 2014). Most of the highly autoreactive T cells are eliminated and a small fraction become suppressive regulatory T cells (Tregs) (Baldwin et al., 2005). Therefore negative selection is crucial to avoid autoimmune diseases.

Regulatory T cells (Tregs) derived from the thymus are also important for self-tolerance, as genetic Treg deficiencies lead to spontaneous development of autoimmune diseases (Brunkow et al., 2001; Ochs et al., 2007; Sakaguchi et al., 2006). The majority of Tregs are CD4⁺FOXP3⁺ T cells (FOXP3 = forkhead box P3) and usually express high levels of CD25 (Ramsdell and Ziegler, 2014; Sakaguchi et al., 2010; Sakaguchi et al., 1995). A mutation in the Foxp3 gene in the scurfy mouse was associated with a severe autoimmune phenotype (Brunkow et al., 2001; Godfrey et al., 1991; Ramsdell and Ziegler, 2014). Consistently, in 2001, the Foxp3 mutation was found responsible for the immune dysregulation in X-linked syndrome (IPEX) in humans (Bennett et al., 2001; Wildin et al., 2001).

Medullary TECs (mTECs) also regulate T cell tolerance by the presentation of a repertoire of self antigens by their MHC molecules for T cells to recognize. mTECs have been shown capable of expressing a wide array of antigens including organ-specific genes like insulin (Jolicoeur et al., 1994). An important gene related to their antigen presentation is the autoimmune regulator (AIRE) (Heino et al., 1999).

Peripheral T cell tolerance
Despite of the mechanisms inducing central tolerance within the thymus, a small fraction of autoreactive T cells are still released into the periphery, however, self-tolerance is usually well maintained in the periphery and autoimmune diseases rarely occur. Despite our limited knowledge, peripheral T cell tolerance is maintained through clonal deletion, anergy and ignorance of autoreactive T cells as well as regulation by Tregs (Ohashi and DeFranco, 2002).

After leaving the thymus, naïve T cells circulate in the blood and the secondary lymphoid organs such as lymph nodes and spleen and are constantly in contact with dendritic cells (DCs) and other antigen presenting cells (APCs) (Chow et al., 2002). Under quiescent situations, APCs constitutively present self-antigens through their MHC class I and class II complexes at a relatively low concentration and with rapid turnover (Eisenlohr et al., 2007; Schlosser et al., 2007). Their interactions with mature naïve T cells that recognize self antigen peptides: MHC complexes at low affinity promote naïve T cell survival and self-tolerance (Eisenlohr et al., 2007; Jenkins, 2013). Upon infection, naïve T cells are able to be activated when they receive three signals from APCs (Hansen and Roche, 2013). Signal one is the activation of TCR through interaction either between TCR/CD4 and peptide: MHC class II, or between TCR/CD8 and peptide: MHC class I on DCs (Fooksman, 2014; Rosette et al., 2001). Signal two is a co-stimulation signal such as the interaction between CD28 on T cells and CD80/86 on DCs (Gimmi et al., 1991). Moreover, DCs can further promote the activation, expansion and differentiation of T cells through cytokine production (signal 3) (Blomgren and Larsson, 1978; Schorle et al., 1991). Of these three signals, signal one and two are necessary for T cell activation (Jenkins, 2013). After activation, T cells can then differentiate into effector T cells which no longer need co-stimulatory signals for their function when encountering their target cells (Jenkins, 2013).

Autoreactive T cells in the periphery could bind to their cognate MHC on APCs at high affinity (signal 1), but without signal 2 and 3, those T cells undergo clonal deletion and anergy (Lamb et al., 1983). For example, T cell clones cultured with cognate virus peptides in vitro without APCs have been found with blocked proliferation when restimulated with antigens together with APCs (Lamb et al., 1983). Accumulated evidence has shown impaired T cell proliferation when T cells are provided with their cognate antigens on APCs but without signaling through CD28 (Boise et al., 1995; Paul, 2013). This decreased proliferation of
autoreactive T cells is called clonal deletion and the unresponsiveness of T cells upon antigen stimulation is termed anergy. Besides clonal deletion and anergy, autoreactive T cells can also literally ignore self-antigens if they circulate but do not bind to self antigens (Goodnow and Ohashi, 2013; Schietinger and Greenberg, 2014).

In the periphery, APCs also play an important role in self-tolerance. First, antigen presentation by APCs without providing co-stimulatory signals (signal 2) and proinflammatory cytokines (signal 3) induces T cell clonal deletion and anergy (Lin et al., 2010). In addition, DCs also take up and present antigens from extracellular matrix and other cells and induce cross-tolerance of autoreactive T cells (Lin et al., 2010).

Despite of the different mechanisms of T cell anergy induction, certain self-reactive T cells are still able to survive. At this stage, FOXP3+ Tregs play an important role in the maintenance of self-tolerance in the periphery, as Foxp3 defects in both human and mouse are associated with spontaneous development of autoimmune diseases (Bennett et al., 2001; Brunkow et al., 2001; Godfrey et al., 1991; Ramsdell and Ziegler, 2014; Wildin et al., 2001). In addition to thymic FOXP3+ Tregs discussed above, FOXP3+ Tregs can also derive from naïve T cells in the periphery upon stimulation (Wing and Sakaguchi, 2010). Tregs are able to suppress autoimmune responses through contact induced apoptosis of targeted responder T cells or APCs, as well as through the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Wing and Sakaguchi, 2010). Recently it has been shown that autophagy is important for Treg lineage stability and function by integrating environmental cues to Tregs (Wei et al., 2016).

**B cell tolerance**

B cells originate from common lymphoid progenitor cells in the bone marrow. The common lymphoid progenitor cells first develop into CD19+ pro-B cells, then become pre-B cells that express a pre-B receptor and eventually transform into naïve B cells that express immunoglobulin M (IgM) (Hardy, 2003; Hardy and Hayakawa, 2001). When mature naïve B cells encounter antigens that express complementary epitopes, their BCRs bind, internalize and digest the antigens and present the antigen peptide on the cell surface through peptide: MHC class II complexes (signal 1). If the antigen peptide:MHC class II complexes on B cells is recognized by activated Th cells from whom they receive Th cell-derived co-stimulatory signals
(signal 2) together with cytokines (signal3), the B cells become activated and differentiate into plasmablasts (Paul, 2013). These cells migrate to primary lymphoid follicles together with Th cells and T follicular helper (Tfh) cells and form germinal centers (GCs) (Shlomchik and Weisel, 2012; Victora and Mesin, 2014). In the GCs, activated B cells undergo extensive proliferation, somatic hypermutation and class switching that allows them to eventually become either memory B cells or plasma B cells that produce high affinity antibodies of different isotypes (Victora and Mesin, 2014).

Similar to T cells, B cell tolerance is regulated both centrally within the bone marrow during development and in the periphery after maturation. B cell tolerance is also regulated by APCs as well. In contrast to T cells, however, autoreactive B cells may not undergo apoptosis in the bone marrow but instead further rearrange their immunoglobulin genes (receptor editing) and if their B cell receptor (BCR) become low/negative self-reactive, the B cells can proceed to maturation (Ohashi and DeFranco, 2002). In addition, it has been shown that the deletion of autoreactive B cells in the bone marrow is not as restrictive as T cells in the thymus. Autoreactive B cells could be found frequently in the spleen and lymph nodes, however, without activation signals from APCs and T cells, most of these autoreactive B cells are anergic and short lived (Goodnow et al., 1989).

Regulatory B cells (Bregs) also play an important role in the maintenance of self-tolerance as reviewed by Rosser et al and Kim et al recently (Kim et al., 2015; Rosser and Mauri, 2015). In 1996, Wolf et al showed that B cell deficient mice were unable to recover from experimental autoimmune encephalomyelitis (EAE) (Wolf et al., 1996). Later, the production of IL-10 by B cells was found critical for the anti-inflammatory role of B cells (Fillatreau et al., 2002; Mauri et al., 2003). Thus Bregs are generally defined as IL-10+ B cells with anti-inflammatory functions. Different IL-10+ Breg populations involved in self-tolerance and control of inflammation have been reported, such as CD19+CD21hiCD23hiCD24hi transitional 2 marginal-zone precursor (T2-MZP) cells, CD5+CD1dhi B (B10) cells, CD19+CD21hiCD23− marginal zone (MZ) B cells (Rosser and Mauri, 2015). In addition to IL-10+ Bregs, other Breg populations with anti-autoimmunity characters have been reported in the last few years. For example, Ray et al showed an IL-10 independent role of B cells in the recovery of mice from EAE through the engagement of T cells and maintenance of Tregs via the glucocorticoid-induced tumor necrosis
factor ligand (GITR) expressed on B cells (Ray et al., 2012). B cell specific deletion of IL-35 in mice also showed impaired recovery from EAE similar as B cell specific IL-10 deficient mice, suggesting an important role of IL-35+ Bregs in self-tolerance maintenance (Shen et al., 2014). Unlike Tregs with the signature FOXP3+ expression, no lineage specific transcription factor could be found within Bregs (Kim et al., 2015; Rosser and Mauri, 2015). Instead, Bregs have been reported at different stages from naïve B cells to plasmablasts and most of the Bregs are mainly found upon stimulation/inflammation (Bodogai et al., 2013; Matsumoto et al., 2014; Rosser and Mauri, 2015). In particular, the discovery of CD20dim or CD20- Bregs and their regulation on T cell-mediated immune responses may help to explain the therapeutic effects of B cell depletion therapy in CD8+ T cell-mediated ITP as will be discussed in Chapter 4 (Bodogai et al., 2013; Matsumoto et al., 2014).

In summary, T cells and B cells are normally non-responsive to self antigens under physiological situations which is maintained by mechanisms such as apoptosis or anergy of autoreactive cells. Despite a great increase of knowledge in the last two decades, our understanding of the mechanisms of self-tolerance still remains incomplete. In ITP and other autoimmune diseases, it is thought that self-tolerance mechanisms are breached and autoreactive T cells and B cells are allowed to induce organ or tissue damages.

**1.3.3.2 Evidence of decreased platelet production and increased destruction in ITP**

ITP is a heterogeneous autoimmune disease and although a failure of maintaining self-tolerance leading to anti-platelet specific immune responses is a complicated process, it has been shown to be related to a number of different immune mechanisms. These include but are not limited to: potential platelet and megakaryocyte defects, contributions from antigen presenting cells (APCs), increases in autoreactive B and T cells, inefficient clearance of those autoreactive immune cells and dysregulation of cytokine production. A schematic view of the pathogenesis of ITP is shown in Figure 1.3.

There are two main reasons for the inadequate number of platelets in the circulation of patients with ITP: decreased platelet production from megakaryocytes and increased peripheral platelet destruction (Cines et al., 2014; McKenzie et al., 2013).
In ITP, impaired platelet production by megakaryocytes was suspected a century ago (Nugent et al., 2009). Indeed, as early as 1915, Frank hypothesized that the thrombocytopenia of ITP may be due to a platelet production defect (Blanchette and Freedman, 1998; Frank, 1915). In the 1940s, light microscopy of the bone marrow from patients with ITP revealed normal or only slightly increased numbers of megakaryocytes, which were disproportional to the degree of thrombocytopenia (Dameshek and Miller, 1946; De La Fuente, 1949; Diggs and Hewlett, 1948; Nugent et al., 2009). In addition, morphological abnormalities of megakaryocytes were observed under light microscopy, including an increased proportion of premature megakaryocytes and decreased platelet producing megakaryocytes, as indicated by scant evidence of platelet formation, lack of granularity, vacuolization of the cytoplasm, and pyknotic nuclei (Figure 1.4) (Dameshek and Miller, 1946; De La Fuente, 1949; Diggs and Hewlett, 1948; Nugent et al., 2009). Electron microscopy (EM) studies confirmed these findings showing swollen mitochondria, distended DMS, condensed chromatin, all of which were consistent with features of apoptosis and this was confirmed by the observation of increased caspase-3 staining in megakaryocytes (Figure 1.4) (Houwerzijl et al., 2004; Nugent et al., 2009; Stahl et al., 1986).

Platelet turnover studies also provided consistent findings by estimating the platelet production rate based on the measurements of radiolabeled platelet survival in vivo with time (with the assumption that the platelet destruction equals production when the platelet count is stable). Under thrombocytopenic conditions such as secondary to blood loss, the megakaryocytes in healthy individuals had the potential of approximately a 10-fold increase in platelet production capacity (Cines et al., 2014). However, this was not observed in patients with ITP, where only decreased or normal platelet production was found (Ballem et al., 1987; Branehog et al., 1975; Harker, 1970; Heyns Adu et al., 1986; Nugent et al., 2009; Stoll et al., 1985).

Increased platelet destruction in ITP was initially suggested by the studies of Harrington et al (Harrington et al., 1951) and this was confirmed by platelet kinetic studies. Using $^{51}$chromium labeled allogeneic platelets, these studies showed that the life span of platelets was about 10 days in healthy humans (Harker and Finch, 1969) and only a few hours to days in patients with ITP (Harker, 1970; Harker and Finch, 1969). The shorter life span of platelets in ITP was also
Figure 1.4: Megakaryocytes from healthy individuals and patients with ITP. Images adapted from Dameshek and Miller, 1946 and Houwerzijl et al., 2004 (Dameshek and Miller, 1946; Houwerzijl et al., 2004).
(A, B) Normal megakaryocytes, magnification ×1000. (C, D) ITP megakaryocytes with rare or no platelet formation, sharp cytoplasmic edges and pyknotic nuclei, magnification ×1000. (E) Normal megakaryoblast showing a lobulated nucleus (N). In the cytoplasm, characteristic demarcation membrane system (asterisks) and normal mitochondria (arrowheads) can be found, magnification × 3000. (F) Megakaryoblast from a patient with ITP in the process of para-apoptosis; N indicates nucleus. The cell has an intact enlarged peripheral margin (large arrow), magnification × 4500. (G) The inset of panel F shows a higher magnification with mitochondria, some of which are slightly swollen (open arrowheads) and others which have completely collapsed cristae which appear as empty vacuoles (asterisks). The enlarged peripheral margin in the cytoplasm is free of organelles (open arrow), magnification × 15 000. (A-D) Light microscopy images of bone marrow megakaryocytes. (E-F) Electron microscopy images of bone marrow megakaryocytes.
confirmed by other groups using $^{51}$ chromium labeled allogeneic platelets or $^{111}$ indium-labeled autologous platelets (Branehog et al., 1975; Branehog et al., 1974; Kernoff et al., 1980). The increased platelet destruction theory was also supported by the effectiveness of splenectomy in raising platelet count as a treatment for ITP (Blanchette and Freedman, 1998; Branehog et al., 1975; Louwes et al., 2001; Nugent et al., 2009).

Despite the clear evidence of decreased platelet production as well as increased destruction in ITP, the factors that trigger the immune responses against self-platelets and megakaryocytes are still elusive.

1.3.3.3 Defects in platelets in ITP

Certain dysfunctions of platelets and megakaryocytes may contribute to the development of ITP. For example, as mentioned in section 1.1.3, healthy human platelets express all the TLRs, which have a protective function against infections (Kapur et al., 2015b). However, the expression of TLR2 and TLR4 on platelets was found to be significantly decreased in both acute and chronic ITP (Wang et al., 2009). The platelets with decreased expression of TLRs might be recognized as ‘abnormal cells’ that leads to their destruction and development of ITP. Alternatively, it may also be possible that the development of ITP is due to increased platelet destruction mediated by activation of TLRs after infections as suggested by animal studies (Aslam et al., 2006; Semple et al., 2007). Perhaps this may help to explain the observation that in children with ITP, febrile illnesses and infections are quite common preceding the development of thrombocytopenia (Cines et al., 2009).

HLA class II complexes are one of the most important proteins upregulated upon infection. Platelets do not normally express HLA class II antigens, however, Boshkov et al reported the expression of HLA-DR on the platelets from a child with active ITP which became negative after remission (Boshkov et al., 1992). Enhanced expression of HLA-DR in ITP was also confirmed by other groups (Semple et al., 1996) and although it was suggested that aberrant HLA-DR expression on platelets may give rise to autoreactive T cells in ITP, further studies are required to confirm this.
Another platelet defect that may potentially trigger ITP is the dysregulation of CD47 expression. CD47 is expressed on virtually all cells (Seiffert et al., 1999) and is a ligand for signal regulatory protein α (SIRPα) on neutrophils, monocytes, and dendritic cells (Seiffert et al., 1999). The interaction between CD47 and SIRPα down-regulates phagocytosis by SIRPα+ cells (Oldenburg et al., 2000; Ravetch and Lanier, 2000; Seiffert et al., 1999). It has been shown that CD47 expression on aged platelets is significantly lower in patients with ITP as compared to healthy controls (Catani et al., 2011), however, whether this enhances the platelet destruction in ITP is still not known.

Senescent platelets are cleared from the circulation continuously and primarily via macrophage-mediated phagocytosis in the spleen (Cines et al., 2014; Clarkson et al., 1986; Harker, 1970). In the last few years, however, a new theory has been proposed that implicates the Ashwell-Morell receptor on liver hepatocytes and macrophages being capable of recognizing asialoglycoproteins (glycoproteins lacking sialic acid) on aged platelets (Grewal et al., 2008; Rumjantseva et al., 2009) and promoting platelet clearance. This Ashwell-Morell receptor mediated platelet clearance might be significantly increased under pathological situations and lead to the development of ITP. In a recent case study reported by Shao et al, a patient with refractory ITP had significantly increased platelet desialylation (Shao et al., 2015) and with oseltamivir phosphate (tamiflu) treatment, a sialidase inhibitor, the desialylation of the platelets gradually decreased and the platelet count of the patient recovered (Shao et al., 2015). Of note, this patient was positive for GPIb-IX antibody, although the antibody titer was unchanged before or after the remission (Shao et al., 2015). Further investigations are still needed to further confirm the relationship among anti-GPIb antibodies, platelet desialylation and ITP development.

Furthermore, increased apoptosis of platelets in ITP may contribute to the development of the disease but this remains controversial. In a study by Catani et al, platelet apoptosis in adult ITP was increased and this was associated with an upregulated T cell stimulation potential of DCs (Catani et al., 2006). However, a study of platelet apoptotic markers in children with ITP by Wang et al showed inconsistent findings (Wang et al., 2010). Different than the findings in adults, the authors examined apoptotic markers on platelets including annexin V, caspase 3, and mitochondrial inner transmembrane potential depolarization, and could not find any significant
changes. In addition, platelets from these paediatric patients with ITP were found to be resistant to apoptosis as suggested by increased CXCR4 expression in the plasma (Wang et al., 2010). These discrepancies between the studies might be due to different pathogenic mechanisms in paediatric versus adult ITP, the stage of ITP or the various therapies the patients may have received.

Overall, defects in platelets could directly increase their phagocytosis by macrophages or indirectly increase platelet destruction through stimulation of DCs and activation of antibody- or T cell-mediated anti-platelet immune responses. This may eventually lead to the development of ITP but more research is required to confirm whether platelet defects are responsible for the autoimmune initiation of the attack against self platelets.

1.3.3.4 Dysregulation of APCs and initiation of adaptive immune responses in ITP

Apart from platelet abnormalities, dysregulation of antigen presenting cells (APC) also seems to be involved in the pathogenesis of ITP. APC is a general term for several different cell types all of which have the capability of taking up and digesting protein antigens into peptides (known as antigen processing) and loading their peptides onto the major histocompatibility complex (MHC) molecules which are the ligands for activating naive T cells (antigen presentation) (Banchereau et al., 2000; Watts, 1997). There are two types of MHC molecules termed MHC class I and class II. While MHC class I molecules are universally expressed on all cells except the RBCs, MHC class II molecules are almost exclusively expressed on APCs and facilitate their function in priming CD4+ T cells (Banchereau et al., 2000). Another important feature of APCs is the expression of various co-stimulation receptors which are important for T cell activation and proliferation such as CD80/86 (Banchereau et al., 2000). APCs include DCs, monocytes (also known as macrophages when activated), B cells and activated epithelial cells etc (Chaplin, 2010). Under normal situations, APCs endocytose exogenous antigens and digest the proteins into peptides with the help of the proteasome in the cytosol or lysosomes. The peptides are then transported into the endoplasmic reticulum (ER) or endosomes for loading onto to MHC class I or class II molecules respectively (Banchereau et al., 2000; Chaplin, 2010). The peptide: MHC complexes are then transported to the cell surface where they can potentially interact with antigen-specific TCRs expressed on T cells (Banchereau et al., 2000; Chaplin, 2010).
Genetic studies of the human leukocyte antigen (HLA) polymorphisms showed an association of certain HLA class II alleles and a predisposition of ITP suggesting a contribution of APCs in initiating ITP. For example, in 1979, Karpatkin et al found an association of HLA-DRw2 on B cells from patients with ITP (Karpatkin et al., 1979) and subsequently, polymorphisms in several HLA class II alleles were found associated with anti-GPIIbIIIa or anti-GPIb-IX antibody production in Japanese patients with ITP (Kuwana et al., 2000; Nomura et al., 1998). However, no association could be found in other Asian populations including patients from China and India (Leung et al., 2001; Negi et al., 2012). In addition polymorphisms in HLA class II alleles were also reported in Italian patients with ITP (Veneri et al., 2002). The differences among these studies could be perhaps explained by the differences in the distribution of HLA alleles in a particular population or may also be due to the heterogeneity of the disease.

DCs are the most important APCs for the initial activation of T cells and have specialized features for antigen processing and presentation. DCs do not only actively macropinocytose extracellular fluid and phagocytose extracellular pathogens through Fc-receptors, but also express a broad variety of anti-microbial pattern recognition receptors (PRRs) to recognize microbial invasions efficiently (Liu and Nussenzweig, 2013). For example, they express nearly all known TLRs for the detection of, for example, bacterial lipopolysaccharides (LPS), lipoproteins, lipopeptides, flagellin, as well as double-stranded RNA of viruses. They also express the intercellular adhesion molecule-3-grabbing nonintegrin (SIGN) family receptors, and multiple C-type lectins including the macrophage mannose receptor (MMR), DEC205 (CD205), and dendritic cell inhibitory receptor 2 (DCIR2, i.e. Clec4a4) and also retinoic acid-inducible gene-I (RIG-I)-like receptors (RLR) etc (Liu and Nussenzweig, 2013). When activated by one or more of these receptors, DCs not only process and present antigens but also increase the expression of co-stimulatory signals and the production of cytokines (Banchereau et al., 2000). This enables DCs to activate naïve T cells and promote T cell proliferation and differentiation. For example, activated DCs regulate the differentiation of naïve CD4+ T cells into Th1, Th2, Th17 cells or suppressive regulatory T cells (Tregs) (Liu and Nussenzweig, 2013). DCs can be divided into at least three groups: plasmacytoid DCs (pDCs), conventional DCs (cDCs), and migratory DCs (mDCs). cDCs can be further divided into CD8α+ and CD8α– cDCs whereas mDCs are also known as myeloid DCs due to their non-lymphatic distribution.
(Gilliet et al., 2008; Liu and Nussenzweig, 2013; Shortman and Liu, 2002). Under normal situations, platelets can be taken up by DCs but the platelet peptide:MHC complexes generated by the DCs do not trigger T cell activation because those platelet peptides would be recognized by T cells as “self” through weak peptide:MHC/TCR interactions and lack of co-stimulatory signals (Banchereau et al., 2000). However, abnormal DC activation, under inflammatory situations may provide simultaneous co-stimulation signal (signal 2) together with signal 1 and lead to the activation of T cells against self-platelet antigens and the development of ITP. In 2006, Catani et al found significantly increased CD86 expression on cultured DCs from the blood and spleens of patients with ITP and this could act as a co-stimulatory ligand through the interaction with CD28 and activates the T and B cells to attack platelets (Catani et al., 2006; McKenzie et al., 2013). When the DCs were pulsed with platelets, those derived from patients with ITP induced higher T cell proliferation responses in vitro (Catani et al., 2006). This suggested an association between increased antigen presenting capability of DCs and an expansion of autoreactive T and B cells in ITP. The increased expression of CD86, together with increased expression of CD80 and HLA-DR on DCs in ITP were also recently confirmed by Zhang et al (Zhang et al., 2015). In addition, these authors found decreased CD205 expression on splenic DCs in ITP which is important for DC antigen presentation and immune tolerance induction (Bozzacco et al., 2007; Fukaya et al., 2012; Zhang et al., 2015; Zhou et al., 2012). The decreased CD205 on DCs in ITP may help to explain the impaired self-tolerance and the Treg deficiency and dysfunction observed in the disease (Liu et al., 2007; Sakakura et al., 2007). Moreover, Saito et al found that ITP was associated with a pDC but not a mDCs deficiency in the peripheral blood, and the pDC deficiency was normalized in responders to H. pylori eradication therapy (Saito et al., 2012).

In addition to DC dysfunction, monocytes have also been shown to be involved in the initiation and promotion of anti-platelet adaptive immune responses in ITP. Monocytes (or macrophages when activated) are another important member of APCs and like DCs, they also contribute to the recognition of foreign antigens and the initiation of adaptive immune responses (Chaplin, 2010). Moreover, activated macrophages are highly phagocytic, engulfing most of the senescent and abnormal cells, and also antibody-opsonized immune complexes, including anti-platelet antibody opsonized platelets in ITP. In ITP, CD16+ (FcγRIIA) monocytes were found
Table 1.2 Summary of the antigen presenting cell (APC) defects in ITP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Major biological function</th>
<th>Response compared to normal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymorphisms in HLA class II</td>
<td>antigen presentation, activation of CD4^+ T cells</td>
<td>Association with HLADRB1<em>0410, HLADRB1</em>0405, HLADRB1*0401</td>
<td>Kuwana et al., 2000 Nomura et al., 1998 Veneri et al., 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nonchanged</td>
<td>Leung et al., 2001 Negi et al., 2012</td>
</tr>
<tr>
<td>plasmacytoid DC</td>
<td>type I interferon production</td>
<td>decreased</td>
<td>Saito et al., 2012</td>
</tr>
<tr>
<td>CD80/86 on DCs</td>
<td>co-stimulation</td>
<td>increased</td>
<td>Catani et al., 2006 Zhang et al., 2015</td>
</tr>
<tr>
<td>CD205 on DCs</td>
<td>immune tolerance induction</td>
<td>decreased</td>
<td>Zhang et al., 2015</td>
</tr>
<tr>
<td>indoleamine 2,3-dioxygenase 1 in DCs</td>
<td>immune tolerance induction</td>
<td>decreased</td>
<td>Zhang et al., 2015</td>
</tr>
<tr>
<td>TLR7 in DCs</td>
<td>antiviral, DC activation</td>
<td>increased</td>
<td>Yu et al., 2011</td>
</tr>
<tr>
<td>CD16^+ monocytes</td>
<td>activation of CD4^+ T cells</td>
<td>increased</td>
<td>Zhong et al., 2012</td>
</tr>
<tr>
<td>DC= dendritic cell</td>
<td></td>
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</table>
increased and could directly interact with T cells to promote the expansion of IFN-γ+ CD4+ T cells while inhibiting the expansion of Tregs (Zhong et al., 2012).

It thus appears that the activation of APCs is a prerequisite for the initiation of anti-platelet specific adaptive immune responses in ITP. Abnormalities in the number, distribution, and function of the APCs as discussed above could all contribute to the development of ITP. A brief summary of the APC defects in ITP is listed in Table 1.2.

1.3.3.5 The role of T cells in ITP

Antigen specific immune responses elicited by T cells are called “cell-mediated” immune responses and together with the “humoral” immune responses elicited by B cells comprise the adaptive immune system (Chaplin, 2010; Paul, 2013). There are two types of TCRs: composed of either a TCR heterodimer of α and β polypeptides or of γ and δ polypeptides (Haas et al., 1993; Huppa and Davis, 2013). The αβ TCR is the major T cell group and the receptor is composed of a variable (V) region (the region responsible for antigen specificity), a constant (C) region, and a transmembrane domain on both chains (Novotny et al., 1986; Paul, 2013). When activated, T cells become effector cells. Effector CD4+ T cells are important for promoting the activation of other T and B cells and regulating immune responses. They can be further divided into different subtypes including Th1, Th2, Th17, Th22, Tfh and Tregs (Paul, 2013). Activated effector CD8+ T cells are usually referred to as CD8+ cytotoxic T lymphocytes (CTLs) (Johnson et al., 1985) and can recognize target cells through TCRs or Fas ligand and kill the target cells through the release of perforin and granzymes (Hamann et al., 1997).

The involvement of T cells in the pathogenesis of ITP has been suspected since the early 1970s even before the discovery of the TCR. In fact, T cell involvement in ITP might date back to the early 1950s when Harrington showed that only 16 of 26 plasma transfusions induced thrombocytopenia suggesting alternative mechanisms (Harrington et al., 1951). In 1970, Piessens et al found increased lymphocyte proliferation in response to autologous platelets in vitro from a patient with ITP as measured by the uptake of tritiated thymidine (Piessens et al., 1970). Consistent findings were reported shortly afterward by Clancy and Wybran et al independently (Clancy, 1972; Wybran and Fudenberg, 1972). These studies provided early evidence of activated lymphocytes in ITP. Clancy also noted inhibited leukocyte migration by
autologous platelets in vitro (Clancy, 1972). Subsequently, accumulated evidence suggested that ITP may be associated with T cell abnormalities. For example, in 1978, Stuart et al enumerated T cells in the blood of both the patients with ITP and their families and found decreased numbers of T cells in some of the families (Stuart et al., 1978). In 1984, Borkowsky and Karpatkin found elevated leukocyte migration inhibition factor from patients with ITP, which is known to be produced by T lymphocytes (Borkowsky and Karpatkin, 1984). In 1987, thymectomy in a 13-year-old girl rescued her myasthenia gravis and ITP suggesting that T cells played a significant role in the disease (Jansen et al., 1987). However the T cell theory of ITP was not defined until 1991 when Semple and Freedman found increased anti-platelet proliferation of peripheral blood mononuclear cells (PBMC) from patients with ITP which was associated with increased IL-2 production (Semple and Freedman, 1991). This study was considered the first clear evidence of the involvement of T cells in the pathogenesis of ITP. Subsequently, the sequencing of complementarity determining region 3 (CDR3) in the β chain of TCRs revealed antigen-induced oligoclonality in T cells from patients with ITP, in particular, non-responders to splenectomy (Fogarty et al., 2003; Shimomura et al., 1996). In 2001, autoreactive T cells were found with specificities against GPIIbIIIa epitopes in ITP (Kuwana et al., 2001) and recently, Zhang et al reported an increased lipid raft aggregation on T cells from patients with ITP, suggesting abnormal T cell signaling in ITP (Zhang et al., 2016). Figure 1.5 shows the potential process of the activation of T cells and development of autoreactive anti-platelet immune responses in ITP.

**CD4+ T cells in ITP**

In addition to the study by Semple et al, increased proliferation of CD4+ T cells in ITP was also confirmed by other groups (Ma et al., 2012; Ware and Howard, 1993). Ma et al also found upregulated Bmi-1 expression associated with increased proliferation and survival of CD4+ T cells (Ma et al., 2012; Yamashita et al., 2008). Altogether, these findings suggest that there is a breach in self-tolerance and an activated anti-platelet CD4+ T cell response in ITP. Further studies on CD4+ T cells and their subsets revealed that in ITP, CD4+ T cells are commonly Th1 polarized. Under physiological situations, Th1 and Th2 constitute the major population of activated CD4+ T cells (Liao et al., 2011; Paul, 2013). Th1 cells are distinguished by their production of IL-2 and IFN-γ and are important for targeting macrophages for clearance of
Figure 1.5: Development of the anti-platelet adaptive immune response. Modified from Coopamah et al (2003). Transfusion Medicine Reviews, 17 (1):69-80, Fig 2. (Coopamah et al., 2003).
(A) Senescent platelets are consistently taken up by macrophages (APCs). Platelet autoantigen peptides are digested and presented by MHC class II on the APC surface. The peptide: MHC class II complex could be recognized by cognate TCRs on CD4+ T cells (signal 1). Signal 1 alone would not activate the CD4+ T cells. When activated, however, APCs increase their expression of co-stimulatory molecules e.g. CD80/86 and CD40 and produce inflammatory cytokines such as type I interferons (IFN) and IL-12. (B) Activation of autoreactive CD4+ T cells. The costimulatory proteins on activated APCs interact with their ligands expressed on CD4+ T cells (signal 2) and together with signal 1, activate autoreactive CD4+ T cells. Both APC and T cell derived cytokines (signal 3) further promotes the activation. (C) Activated autoreactive CD4+ T cells differentiate, proliferate, and produce cytokines in favor of a Th1 responses including increased production of IL-2, IFN-γ, IL-17, IL-22, and decreased IL-10 and TGF-β. The activation of autoreactive CD4+ T cells then drives the activation of autoreactive B cells (D) and autoreactive CD8+ T cells (E). The activated B cells differentiate into plasma B cells which produce anti-platelet antibodies and the CD8+ T cells differentiate into CTLs which can directly kill platelets and megakaryocytes.
intracellular bacteria and amplification of cell-mediated immune responses (Liao et al., 2011). Th2 cells on the other hand, are characterized by the production of IL-4, IL-13 and IL-5 and are important for immunity against extracellular helminthes and initiating the production of IgE (Prussin et al., 2010). Both Th1 and Th2 cells are often called helper T cells but they suppress the proliferation and differentiation of each other (Paul, 2013). It is now well established that an imbalance of Th1/Th2 cells is associated with autoimmune diseases. Work by Semple et al showed increased serum IL-2, IFN-γ and/or IL-10, not IL-4 nor IL-6 in patients with ITP suggesting an early activation of CD4⁺ T cells and a Th1 polarized response (Semple et al., 1996). Th1 polarized T cell responses in ITP were further confirmed by increased IFN-γ⁺CD4⁺/IL-4⁺CD4⁺ (Th1/Th2) ratios in patients (Stasi et al., 2007; Wang et al., 2006) and these studies suggest that CD4⁺ T cell activation and Th1 responses may be responsible for the activation of CD8⁺ T cell- and antibody-mediated platelet destruction in ITP. Alternatively, it is possible that the Th1/Th2 imbalance is a consequence of thrombocytopenia as it has been shown that platelets are a source of multiple cytokines and thrombocytopenia is associated with their decreased levels which may be important for the Th1/Th2 balance (Feng et al., 2012; Khan et al., 2005). In addition, there is also a possibility that thrombocytopenia secondary to infection leads to a decreased level of soluble CD40L in vivo which induces a Th1 polarized immune response (Kuwana et al., 2003). Taken together, it appears that the most consistent T cell abnormality in ITP is a Th1 cytokine skewing (Figure 1.5). However, other T cell subsets have also been shown involved.

**Th17 cells in ITP**

Th17 cells are a relatively minor CD4⁺ T cell population characterized by their production of their signature cytokines including IL-17 (IL-17A and IL-17F), IL-21, and IL-22. Th17 cells are induced when both IL-6 and transforming growth factor (TGF-β) are present and when IL-4 and IL-12 are absent (Paul, 2013). The major function of Th17 cells is to enhance neutrophil generation against extracellular bacteria and fungi infection under physiological situations (Isailovic et al., 2015; Schwarzenberger et al., 1998). However, Th17 cells have been found to play a proinflammatory role in the pathogenesis of several autoimmune diseases including ITP (Bettelli et al., 2006; McKenzie et al., 2013; O'Shea, 2013; Wilson et al., 2007). In 2009, Zhang
et al first reported increased Th17 cells in the peripheral blood of patients with ITP and this correlated with an increase in Th1 populations (Zhang et al., 2009). However, quantification of Th17 cytokines including IL-17A, TGF-β and IL-6 in patient plasma showed comparable levels to controls. Two years later, Cao et al reported no significant difference of Th17 populations in peripheral blood mononuclear cells (PBMC) between patients with ITP and healthy individuals (Cao et al., 2011). In the same year, however, a larger prospective study by Rocha et al, showed a 5 fold increase of IL-17A in the plasma of patients with ITP and increased expression of IL-23, IL-6, IL-1, IL-2, IL-12 but not TGF-β (Rocha et al., 2011). The increased Th17 and Th17 cytokines in active ITP were then confirmed by several other studies (Hu et al., 2012; Hu et al., 2011; Ji et al., 2012; Ye et al., 2015). Furthermore, the levels of IL-17, IL-22 and IL-23 were found decreased in patients who responded to treatment (Cao et al., 2012; Li et al., 2015c; Ye et al., 2015). In general, IL-17 is increased in active ITP and appears to be positively correlated with Th1 immune responses and negatively correlated with the remission of the disease. However, it is worth noting that there are some discrepancies in the literature. While this may be related to limited sample sizes and the sensitivity of quantitative studies, further investigations are needed to better understand the role of Th17 cells in ITP.

**Th22 cells in ITP**

Th22 cells are another type of CD4+ T cell subset that may be involved in the development of ITP. Th22 cells have both pro- and anti-inflammatory effects under different circumstances but in combination with Th17 cells, they are overtly pro-inflammatory. In the study by Cao et al, although the authors did not observe any significant Th17 levels, they did find increased IL-22 in the plasma from patients with active ITP and of interest, they found that the IL-22 was decreased after treatment (Cao et al., 2012). In another study by Hu et al, both Th17 and Th22 cells were found increased in ITP together with an increase of IL-22 in patients’ plasma (Hu et al., 2012). In both studies, the increased IL-22 correlated with increased Th1 responses suggesting the IL-22 produced by Th22 cells promoted T cell differentiation towards Th1 which further promotes the development of ITP (Cao et al., 2011; Hu et al., 2012).

**Regulatory T cells (Tregs) in ITP**
Tregs are a subset of T cells which are important for the maintenance of self-tolerance and suppression of both T cell and B cell responses (Ramsdell and Ziegler, 2014). Tregs have been shown to suppress immune responses through contact-dependent mechanisms and the production of soluble factors, such as the cytokines TGF-β and IL-10 (Buckner, 2010; Sakaguchi et al., 2010; Vignali et al., 2008). Treg deficiency is associated with many autoimmune diseases (Bennett et al., 2001; Brunkow et al., 2001; Godfrey et al., 1991; Ramsdell and Ziegler, 2014; Wildin et al., 2001).

Many studies have now confirmed that in patients with active ITP, there is a significant deficiency/dysfunction of Tregs (Reviewed in McKenzie et al, 2013). Of interest, the peripheral deficiency of Tregs could be restored after several different types of treatments such as dexamethasone, rituximab, TPO receptor agonists (TPO-RAs) or IVIg (Bao et al., 2010; Ling et al., 2007; Stasi et al., 2008; Yu et al., 2008). These results suggest that perhaps the rise in platelet counts by the treatments may be responsible for affecting Treg numbers in vivo. The decreased number of Tregs has also been shown to be associated with impaired cross-talk between Tregs and DCs, for example, due to decreased expression of the immunomodulatory enzyme indoleamine 2,3-dioxygenase 1 (IDO1) on DCs (Catani et al., 2013).

The Treg abnormalities in ITP and other autoimmune diseases may be due to several reasons (Buckner, 2010; Vignali et al., 2008). For example, Treg deficiency may occur when target cells express inadequate cell surface markers that are involved in contact-dependent suppression such as cytotoxic T lymphocyte antigen 4 (CTLA-4). Although genetic studies did not find any associations between CTLA-4 polymorphism and a predisposition to ITP, plasma CTLA-4 levels have been found decreased in acute ITP and elevated after treatment (Li et al., 2011; Pavkovic et al., 2003; Radwan and Goda, 2015; Zhu et al., 2015). In addition, it was found that CTLA-4-Ig stimulated DCs from patients with ITP to promote formation of Tregs in vitro (Xu et al., 2012). Alternatively, the Treg deficiency may be due to a dysfunction of Treg populations, for instance, a deficiency in the transcription of FOXP3 or in the production of suppressive cytokines, such as TGF-β or IL-10 (Catani et al., 2013; Chen et al., 2014; Li et al., 2015a). In addition, the local milieu may play a role in the dysfunction of Tregs, as they may be significantly influenced by different cytokines such as IL-2, IL-12, and IL-21 (Andersson et al., 2002; Clough et al., 2008; King and Segal, 2005; Tesse et al., 2012; Thornton et al., 2004).
The imbalance of Th17/Treg in ITP

In addition to the independent role of either Th17 or Tregs, the Th17/Treg ratio is also considered as an indicator of the immune balance, because of the dichotomy in the generation of Th17 cells and Tregs. On the one hand, the presence of TGF-β with IL-6 and IL-23 favors Th17 differentiation from naïve CD4+ T cells and inhibits Treg development (Afzali et al., 2007; Bettelli et al., 2006; Mangan et al., 2006). On the other hand, TGF-β without IL-6 favors Treg differentiation and FOXP3 transcription which in turn inhibits Th17 development (Afzali et al., 2007; Bettelli et al., 2006; Mangan et al., 2006). The Th17/Treg balance is an important regulator of Th1/Th2 responses, and a Th17/Treg shift towards Th17 predominance is associated with inflammation and autoimmune diseases (Afzali et al., 2007).

The Th17/Treg ratio may be a more sensitive indicator to distinguish ITP severity compared with changes in either Th17 or Tregs. For example, in the study by Ji et al, patients with ITP were divided into two groups: an observational group whose platelet counts were above 30×10^9/L without active bleeding, and those in a treatment required group, whose platelet counts were lower or with active bleeding symptoms (Ji et al., 2012). Significant skewed Th17/Treg ratios towards Th17 were found in the latter group (Ji et al., 2012). In addition, a recent study by Yu et al, using an inhibitor of the notch signaling pathway DAPT, showed that the Th17/Treg ratios were decreased in a dose-dependent manner (Yu et al., 2015). Their study not only confirmed the role of the notch signaling pathway in Th17/Treg regulation, but also provided a potential immunotherapy strategy for ITP.

As is shown in Figures 1.3 and 1.5, CD4+ T cells play a central role for the activation and regulation of both antibody- and CTL- mediated immune responses. Abnormalities in the distribution of different subsets of CD4+ T cells as well as biased cytokine production leads to a breach of self-tolerance and development of autoreactive plasma B cells and CTLs that eventually can mediate the destruction of platelets and/or megakaryocytes.

CD8+ T cells in ITP

Beside CD4+ T cells, another important type of T cell subset participating in the pathogenesis of ITP is CD8+ T cells. When naïve CD8+ T cells are activated after their TCR
recognizing specific peptide:MHC class I complexes on target cells, they begin to express granule effector molecules and Fas Ligand (FasL) and differentiate into effector cells called cytotoxic T lymphocytes (CTLs) (Cui and Kaech, 2010; Hanabuchi et al., 1994). In contrast to CD4+ T cells whose primary function is to enhance or inhibit immune cells, CTLs can directly induce apoptosis of target cells through their granule-mediated pathway or death receptor pathway (Cui and Kaech, 2010; Kelso et al., 2002). In granule-mediated cell death, CTLs release cytotoxic granules that contain apoptosis-inducing proteins such as perforin and granzymes A and B into target cells (Lieberman, 2010; Lieberman, 2013; Masson et al., 1990; Metkar et al., 2002). In the death receptor pathway, CTLs express Fas ligand (FasL) that recognizes Fas expressed on abnormal cells and triggers programmed cell death of the target cells (Fisher et al., 1995; Lieberman, 2010). The target cells subsequently break up into small apoptotic bodies that are phagocytosed by macrophages (Kerr et al., 1972). The classical in vitro assay to measure antigen-specific CTL function is called a cytotoxicity assay. Purified CD8+ T cells (effectors) are co-cultured in vitro with radioactive or fluorescence labeled target cells that express cognate peptide:MHC class I complexes for various times and then the apoptosis/death of the target cells is measured (Guo et al., 2014; Kay et al., 1977; Olsson et al., 2003). This assay has been instrumental in defining the role of CTLs and has shown that they are important in anti-virus and anti-tumor immunity and also in the pathogenesis of a number of autoimmune diseases, such as diabetes, multiple sclerosis and ITP (Faustman and Davis, 2009; Li et al., 2007; Olsson et al., 2003; Saxena et al., 2011; Spits et al., 1986; Zhang et al., 2006). In addition, a genetic deficiency in the granule-mediated T cell killing pathway can lead to increased incidence of spontaneous lymphomas and a deficiency in the Fas-FasL pathway is associated with a predisposition to autoimmune diseases (Lieberman, 2013). Thus, the activation and function of CTLs are important parameters of adaptive immune responses.

In 2003, Olsson et al showed increased cytotoxic activity of T cells against autologous platelets in patients with active ITP, suggesting for the first time the involvement of CD8+ T cell mediated platelet lysis in the pathogenesis of the disease (Olsson et al., 2003). In addition to platelet destruction, Li et al demonstrated that CTLs could also induce megakaryocyte para-apoptosis in ITP (Li et al., 2007). A gene expression/pathway analysis by Sood et al revealed the activation of several death receptor pathways e.g. JAK/STAT, all of which suggest CD8+ T cell
activation and cell-mediated cytotoxicity in ITP (Sood et al., 2008). Although the total number of CD8\(^+\) T cells seemed to be comparable between patients with ITP and healthy individuals, the IFN-\(\gamma\)^+CD8\(^+\)/IL-4^+CD8\(^+\) ratio was found significantly higher in active ITP, and decreased after treatment, indicating an imbalance of subpopulations of CD8\(^+\) T cells in the pathogenesis of the disease (Stasi et al., 2007). Furthermore, Audia et al investigated the spleens from patients with ITP who were rituximab nonresponders and found increased IFN-\(\gamma\)^+CD8\(^+\) T cells together with increased CD27 CD28^−CD8\(^+\) effector memory T cells (Audia et al., 2013). In addition, the authors found increased expression of HLA-DR and granzyme B on CD8\(^+\) T cells, indicating increased CD8\(^+\) T cell activation in ITP (Audia et al., 2013). Taken together, it appears that the activation and clonal expansion of autoreactive CD8\(^+\) T cells also play a significant role in the development of ITP, possibly due to the activation of APCs and upregulation of MHC class I/co-stimulation molecules or the activation of CD4\(^+\) T cells favoring Th1 and Th17 cells. The autoreactive CD8\(^+\) T cells could induce direct lysis of platelets and megakaryocytes, which process is known as CD8\(^+\) T cell-mediated ITP.

In summary, dysregulation of T cells play an important role in ITP (Figure 1.5). T cell abnormalities in ITP include increased proinflammatory Th1, Th17 and Th22 cells, decreased Th2 and CD4\(^+\) Treg cells, as well as increased IFN-\(\gamma\)^+CD8\(^+\) and decreased IL-4^+CD8\(^+\) T cells. These abnormalities are associated with the activation of APCs and moreover, the activation of CD4\(^+\) T cells promotes the activation of autoreactive B cells in ITP.

1.3.3.6 Activation of B cells and anti-platelet antibody production in ITP

Antibody-mediated ITP was the earliest mechanism discovered in the pathogenesis of ITP (Harrington et al., 1951) and a failure of B cell tolerance maintenance may also give rise to autoreactive B cells which produce anti-platelet antibodies.

In 1951, Harrington showed that the transfusion of plasma from patients with ITP into healthy volunteers induced transient thrombocytopenia in most recipients, clearly indicating an ITP inducing factor in the plasma (Harrington et al., 1951). In 1965, Shulman et al showed that the thrombocytopenia-inducing factor in the plasma of patients with ITP was quantitatively and qualitatively found in the gammaglobulin fraction of plasma, suggesting that the ITP inducing factor was IgG (Shulman et al., 1965). In 1972, using rabbit anti-human IgG to absorb anti-
platelet antibodies in the sera, Karpatkin et al confirmed that there were anti-platelet IgG antibodies in the plasma of approximately 65% of patients with ITP (Karpatkin et al., 1972). Subsequently, Hymes et al examined the isotype of IgG in antibody positive ITP patients, and found IgG1 the most abundant (Hymes et al., 1980). It was estimated that about two thirds of patients with ITP have detectable anti-platelet antibodies (Chan et al., 2003; Karpatkin et al., 1972). The specificity of anti-platelet antibodies in ITP was not clear until 1982 when studies using platelets from healthy donors and GPIIbIIIa deficient patients with Glanzmann thrombasthenia showed that the majority of autoantibodies in ITP were against platelet GPIIbIIIa (van Leeuwen et al., 1982). Later, anti-platelet IgM and IgA antibodies were also reported in ITP (Liel et al., 2014) and several studies have now confirmed that the majority of anti-platelet antibodies in ITP target GPIIbIIIa and GPIb-V-IX, while some antibodies can also react with GPIV, α2β1, P-selectin (CD62P), αvβ3, glycosphingolipids and cardiolipin (Liel et al., 2014).

The production of autoreactive anti-platelet antibodies by plasma B cells and its regulation may involve multiple factors such as APC activation, enhanced Th1 and Th17 responses, Treg dysfunction/deficiency as well as defects within B cells and related cytokines (Paul, 2013). For example, B-lymphocyte stimulator (BlyS) produced by APC was found significantly increased in the circulation of ITP patients, which is important for promoting B cell survival, proliferation and differentiation (Yu et al., 2011). In addition, CD19+CD24hiCD38hi human regulatory B cells (Bregs) that have suppressive function for CD4+ T cells were found to be decreased in ITP (Li et al., 2012b). This was found to be associated with decreased expression of IL-10 upon stimulation in vitro, which was rescued in patients who responded to treatment, suggesting that like the Treg deficiency observed in ITP, a Breg deficiency is also involved in the development of the disease (Li et al., 2012b).

1.3.3.7 Anti-platelet antibody-mediated ITP

Antibodies, also known as soluble immunoglobulins, are roughly Y-shaped molecules consisting of two identical heavy chains and light chains which are produced by mature B cells and plasma B cells. The two arms of the Y are two identical variable regions (V regions), each of which is composed of V domains from a heavy and a light chain. The V region determines the antigen specificity and affinity of the Ig while the trunk of the Y is the constant region (C
region) and determines the isotype of the Ig (Murphy et al., 2012; Paul, 2013; Williams and Barclay, 1988). The protease papain cleaves the antibody into three pieces: two antigen-binding fragments (Fab) that contain the antigen binding V regions, and a crystallized fragment (Fc) which is part of the C region capable of interacting with Fc receptors (FcRs) (Kalmanson and Bronfenbrenner, 1942; Petermann, 1946; Schirrmacher et al., 1975). Another protease pepsin, on the other hand, cuts the antibody into an F(ab’)_2 fragment in which the two arms of the antibody remain linked and several fragments of the remaining constant region (Deutsch et al., 1946; Kirkham and Schroeder, 1994; Petermann and Pappenheimer, 1941). The antigen binding site of each V region is dependent on the three CDRs, CDR1, CDR2, and CDR3 (Kirkham and Schroeder, 1994). Similar to TCRs, it is estimated that up to 10^{12} different antibodies can be made in an individual through VDJ recombinations, heavy chain and light chain combinations, class switching and B cell hypermutation (Schroeder et al., 1998; Schroeder et al., 1995). In contrast, whereas TCRs only recognize antigen peptides presented through MHC class I or class II complexes, the antibody molecule can directly bind to antigens that bear either peptides (linear determinant) or conformational determinants in the native antigen (Murphy et al., 2012; Paul, 2013).

The immunoglobulins produced by plasma B cells contribute to antigen specific immunity in mainly three different ways: neutralization, opsonization, and complement activation (Bottazzi et al., 2010; Michaelsen et al., 1991). Neutralization refers to the binding of antibodies to antigens and preventing the penetration of antigen into cells (Bottazzi et al., 2010). Opsonization refers to the phagocytosis enhancement of antigen-antibody complexes by FcR expressing cells such as macrophages (Bottazzi et al., 2010). The activation of the complement system is triggered by the binding of the complement C1q fragment to IgG which ultimately leads to cell death by osmotic lysis (Bottazzi et al., 2010). Under physiological situations, there might be a few self-reactive B cells but these B cells and the antibodies they produce are not pathogenic, due to their low-affinity against self-antigens or lack of support from helper T cells (Paul, 2013).

Autoreactive IgG anti-platelet antibodies could induce the development of ITP in several different ways, including increasing platelet destruction, decreasing platelet production, or interfering with platelet function. Of these mechanisms, antibody-mediated platelet destruction
Table 1.3 Summary of Fc receptor expression patterns and functions. Modified from Paul W. E.. Fundamental Immunology (Philadelphia : Wolters Kluwer Health/Lippincott Williams & Wilkins, c2013) Figure 24.1. (Paul, 2013).

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Expression</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>FcγRI</td>
<td>Macrophages, Neutrophils, Eosinophils, DCs</td>
<td>Activation, enhance effector response</td>
</tr>
<tr>
<td>FcγRIIA</td>
<td>Macrophages, Neutrophils, Mast cells, Eosinophils, Platelets, DCs</td>
<td>Activation, effector activation</td>
</tr>
<tr>
<td>FcγRIIB</td>
<td>Macrophages, Neutrophils, Mast cells, Eosinophils, DCs, B cells</td>
<td>Inhibition, set threshold for effector cell activation, maintain tolerance</td>
</tr>
<tr>
<td>FcγRIIIA</td>
<td>Macrophages, Basophils, Mast cells, NKs, DCs, B cells</td>
<td>Activation, dominant pathway for effector activation by IgG</td>
</tr>
<tr>
<td>FcγRIIIB</td>
<td>Neutrophils</td>
<td>Decoy, synergize with FcγRIIIA</td>
</tr>
<tr>
<td>FcεRI</td>
<td>Basophils, Mast cells, Eosinophils, platelets, DCs</td>
<td>Activation, degranulation, allergy</td>
</tr>
<tr>
<td>FcαRI</td>
<td>Macrophages, Neutrophils, Eosinophils</td>
<td>Activation, effector cell activation</td>
</tr>
</tbody>
</table>
and enhanced clearance in vivo is considered the most important mechanism of thrombocytopenia in ITP and has been well studied (Provan et al., 2010). It can be further divided into Fc receptor (FcR) mediated phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells, complement activation-induced platelet lysis, and direct platelet fragmentation.

**Clearance of antibody-bound platelets by Fc receptors**

Fc receptor (FcR) mediated phagocytosis seems to be the dominant mechanism of platelet destruction in ITP. FcRs refer to a family of receptors that recognize the Fc fragments of immunoglobulins and the receptors for IgG are termed Fcγ receptors (FcγR) (Fridman, 1991). Both DCs and monocytes express all four types of FcγRs, including the activating receptors FcγRI, FcγRIIA, FcγRIIIA (FcγRIV in mouse) that promote phagocytosis, and the inhibitory receptor FcγRIIB (Ravetch and Nimmerjahn, 2013) that suppresses phagocytosis. As the Fc portion of IgG antibodies could be recognized by multiple FcγRs with different affinities, the overall response of DCs or monocytes is dependent on the activation/inhibition ratio (Ravetch and Nimmerjahn, 2013). A summary of the FcRs is listed in Table 1.3.

The importance of FcγR dependent phagocytosis in ITP is supported by several observations. Early evidence came indirectly from the observation that splenectomy could significantly increase the platelet counts in most of the patients with ITP (still considered one of the “gold standard” therapies) (Blanchette and Freedman, 1998; Hirsch and Dameshek, 1951). It was found that platelet-associated IgG was increased after splenectomy in some patients but patients could still maintain remission, suggesting that remission is not necessarily due to decreased antibody production but rather the absence of platelet destruction in the spleen (Luiken et al., 1977; McMillan, 1981). In 1974, Handin et al showed that antibody-coated platelets could be phagocytosed by autologous peripheral leukocytes in vitro, in particular by neutrophils, suggesting the phagocytosis of antibody-platelet complexes in vivo in ITP (Handin and Stossel, 1974). Later, the effectiveness of IVIg in ITP management and related studies further improved our understanding of the FcγR mediated phagocytosis of platelets in the pathogenesis of the disease. In 1981, Imbach et al reported that IVIg was effective in pediatric chronic ITP, and in the following two years Fehr et al and Newland et al showed similar findings in adult ITP (Fehr et al., 1982; Imbach et al., 1981; Newland et al., 1983). It was
suspected that the high dose IVIg raised platelet counts in ITP through competitive binding to FcRs leading to decreased Fc dependent phagocytosis of anti-platelet IgG-coated platelets. This was supported by the study of Clarkson and colleagues in 1986 showing that infusion of purified monoclonal antibody against both FcγRII and FcγRIIIA into patients could induce an immediate increase of platelet counts and ameliorate ITP (Clarkson et al., 1986). In an analogous set of observations, intravenous anti-D immunoglobulin (IV anti-D) infusions were also found to increase platelet counts in Rh⁺ ITP patients, but not in Rh⁻ patients, suggesting anti-D opsonized RBC may competitively block FcR-mediated platelet phagocytosis in ITP (Lazarus and Crow, 2003; Salama et al., 1984; Salama et al., 1986). It is now well accepted that FcR-mediated phagocytosis of antibody-bound platelets is a major mechanism responsible for the increased platelet destruction in ITP and spleen is the major site for the attack. This process in the spleen appears to be blocked by therapies such as splenectomy, IVIg, IV anti-D and purified anti-FcγR antibodies.

It is worth noting that although antibody-mediated platelet destruction is considered the major mechanism and the spleen is considered the major site for platelet destruction in patients with ITP, more than one third of patients fail splenectomy and this suggests that the immune response against platelets can linger outside of the spleen (Provan et al., 2010).

In the last decade, there have been further studies on different subtypes of FcRs in ITP. In 2008, Asahi et al found decreased expression of the inhibitory FcγRIIB on monocytes from H. pylori positive Japanese patients with ITP compared with H. pylori negative patients suggesting a decreased inhibition of macrophage activation in some patients (Asahi et al., 2008). Later, Satoh and colleagues found a genetic polymorphism of the FcγRIIB gene in patients with ITP (Satoh et al., 2013). However, this could not be confirmed in another study (Xu et al., 2010). Overall, the increased platelet destruction in ITP may be a consequence of the increased activation/inhibition ratio of the differing activating and inhibitory FcR, e.g. increased FcγRIIIA activation with reduced FcγRIIB suppression. Further studies are still needed to confirm the role of FcγRIIB receptors in ITP.

The role of natural killer (NK) cells in the development of ITP is controversial. In the study by Engelhard et al, NK cell activity was higher than normal in a patient with ITP, and was
downregulated by IVIg therapy (Engelhard et al., 1986). In 1991, Semple et al showed that PBMC from patients with ITP have decreased NK cell cytotoxic activity against the NK-sensitive cell line K562 suggesting defective NK cell activity in ITP (Semple et al., 1991). Related to this, Soubrane et al found that the decreased NK function in vitro could be rescued by the injection of soluble anti-CD16 (FcγRIII) antibody in a patient with secondary ITP (HIV associated) (Soubrane et al., 1993). In apparent contrast, however, Garcia-Suarez et al found increased numbers of CD56+CD3− NK cells in patients with chronic ITP but they did not address cytotoxicity (Garcia-Suarez et al., 1993). Later it was shown that the polymorphism of a pseudogene FcγRIIc in ITP was associated with a significantly increased expression of FcγRII on NK cells and NK activation (Breunis et al., 2008). NK cells also express FcγRIII and therefore are capable of recognizing IgG-coated targets to induce lysis of the target cells, a process known as antibody dependent cellular cytotoxicity (ADCC) but few studies have examined this in ITP. More studies are required to sort out these observations but it is possible that the FcRs on CD16+ NK cells are occupied by IgG-platelet complexes, leading to destruction of platelets and/or megakaryocytes by ADCC.

1.3.3.8 Other pathways of antibody-mediated ITP

Activation of the complement system may also be involved in the platelet destruction in ITP. In addition to recognition by FcR, the Fc portion of antibodies can also be recognized by the C1q component of complement leading to activation of the complement cascade and eventual lysis of the antibody bound targets. In 1979, Cines et al reported increased C3 on the surface of platelets in a subpopulation of patients with ITP suggesting activation of the complement system in ITP. The elevated C3 in ITP was confirmed by other groups with additional findings of increased C4 on the platelet surface (Winiarski and Holm, 1983). More recently, Peerschke found increased complement activation capacity (CAC) in the plasma of patients with ITP and this was associated with a decreased circulating absolute IPF and thrombocytopenia (Peerschke et al., 2010). These studies suggested that complement mediated platelet destruction may have a role to play in ITP.

It has also been proposed that in certain forms of secondary ITP such as the ITP associated with HIV infections, autoreactive anti-platelet antibodies may induce platelet destruction by
directly binding the platelet and inducing reactive oxygen species (Cines et al., 2014; Nardi et al., 2001; Nardi et al., 2007). Perhaps related to this, the oxidative stress level was found significantly increased in pediatric chronic ITP (Zhang et al., 2011). Through gene expression analysis of whole blood, they found that the oxidative stress-related pathways were among the most significantly activated pathways in chronic ITP, which was different from acute ITP in children. Moreover, the authors found that the overexpression of vanin-1, an oxidative stress sensor in epithelial cells was positively associated with disease progression suggesting potential prognostic value of vanin-1 in the clinical management of ITP (Zhang et al., 2011).

Increased platelet activation induced by autoreactive antibodies in ITP has also been proposed but remains disputable (Cines et al., 2014). In 1987, Sugiyama et al identified an antibody in a patient with ITP that induced significant platelet aggregation (Sugiyama et al., 1987) and Yanabu et al subsequently showed the presence of anti-platelet CD9 IgG autoantibodies in ITP plasma capable of activating platelets in an FcγRII-dependent manner (Yanabu et al., 1993). More recently, Urbanus et al demonstrated that anti-GPIbα antibodies significantly increased platelet activation via the translocation of GPIbα into lipid rafts and activation of FcγRIIa in a single case of ITP (Urbanus et al., 2013).

In addition to their binding to platelets, autoreactive antibodies also impair platelet production through their interactions with megakaryocytes in the bone marrow of patients with ITP (Cines et al., 2014). As is mentioned above, it has been noted that in ITP there is an increased apoptosis of megakaryocytes and a decreased platelet production from megakaryocytes (Dameshek and Miller, 1946; Houwerzijl et al., 2004). In 2003, Chang et al showed that plasma from ITP patients as well as purified anti-platelet monoclonal antibodies could inhibit the yield of megakaryocytes from umbilical cord blood mononuclear cells in vitro (Chang et al., 2003). McMillan et al further showed that anti-platelet antibodies were not only able to inhibit megakaryocytopoiesis but also decreased ploidy of megakaryocytes, suggesting impaired megakaryocyte maturation in patients with ITP (McMillan et al., 2004). In addition, Balduini showed that anti-GPIbα but not anti-GPIIbIIIa antibodies inhibited pro-platelet formation from umbilical cord blood derived megakaryocytes in vitro, suggesting an additional mechanism that contributes to the immune thrombocytopenia in ITP (Balduini et al., 2008). Of interest, however, work in this thesis suggests that in vivo, anti-platelet antibodies may not have
a significant role to play in megakaryocyte destruction in passive ITP (Chapter 2). Reasons for
the differences between the in vitro and in vivo megakaryocytes results are not clear.

In conclusion, ITP is a heterogeneous disease and multiple compartments of the immune
system all contribute to the development of platelet and megakaryocyte destruction. The major
immune mechanisms involved are shown in Figure 1.3. Initially, there could be intrinsic defects
in platelets and megakaryocytes or environmental triggers that promote increased clearance of
platelets by activated APCs and enhanced antigen-presentation of platelet epitopes under
inflammatory situations. This could trigger the activation of adaptive autoimmunity against
platelets. Activated autoreactive Th1 and Th17 CD4+ T cells and impaired Tregs may promote
both antibody-mediated and CD8+ T cell-mediated ITP. While CD8+ T cells mediate ITP
through direct platelet lysis and/or megakaryocyte apoptosis induction, the antibody-mediated
ITP involves several mechanisms including Fcγ receptor mediated phagocytosis of platelets,
impaired production of platelets, as well as increased activation and consumption of platelets.

1.3.4 Treatment of ITP

A few years ago, the IWG published a guideline for the diagnosis and management of
ITP based on current evidence from the literature and expert opinions (Provan et al., 2010). The
review here is based on that guideline with updated evidence from clinical trials in the last few
years. A therapeutic summary is shown in Table 1.4. A principle recommended in the guideline
was that treatment options should be considered based on the symptoms and needs of the
patients, not on their platelet counts. Two of the treatments are related to my PhD study, i.e. the
allogeneic platelet transfusions and B cell depletion therapy, will be further discussed in future
sections.

Treatment of adult ITP

Patients with platelet counts above 50×10^9/L should be under observation without any
treatment unless there is active bleeding or under special circumstances that demand a safe level
of platelet counts such as surgery or anticoagulation therapy (Provan et al., 2010). Patients with
platelet counts below 50×10^9/L but above 20×10^9/L are not necessarily treated without
significant bleeding symptoms. Patients with platelet counts below 20×10^9/L, however, have a

<table>
<thead>
<tr>
<th>Clinical situation</th>
<th>Therapy option</th>
<th>Recommended dose</th>
<th>Approximate response rate</th>
<th>Toxicities</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-line</td>
<td>Prednis(ol)one</td>
<td>0.5-2 mg/kg/d</td>
<td>70%-90% of patients respond initially. 20-50% of patients have sustained platelet count.</td>
<td>Vary with length of administration: mood swings, weight gain, anger, anxiety, insomnia, Cushingoid faces, dorsal fat, diabetes, fluid retention, osteoporosis, hypertension, GI distress and ulcers, etc.</td>
</tr>
<tr>
<td></td>
<td>Dexamethasone</td>
<td>40 mg daily for 4 d, every 2-4 wk for 1-4 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methylprednisolone</td>
<td>30 mg/kg/d for 7 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV anti-D</td>
<td>50-75 μg/kg</td>
<td>Initial response rate similar to IVIg, typically last 3-4 wk but may persist for months in some patients.</td>
<td>Hemolytic anemia (dose-limiting toxicity), fever/chills.</td>
</tr>
<tr>
<td></td>
<td>IVIg</td>
<td>0.4 g/kg/d for 5 d or infusions of 1 g/kg/d for 1-2 d</td>
<td>Initial response rate up to 80%, usually transient; occasionally persists for months.</td>
<td>Headaches, transient neutropenia, renal insufficiency, aseptic meningitis, thrombosis, flushing, fever, chills, fatigue, nausea, diarrhea, blood pressure changes and tachycardia.</td>
</tr>
<tr>
<td>Second-line</td>
<td>Rituximab</td>
<td>375 mg/m²/wk×4</td>
<td>Initial response rate 60-80%, 2-year response rate above 40%, 5-year response rate about 20%.</td>
<td>Fever/chills, rash, or scratchiness in throat, serum sickness and (very rarely) bronchospasm, anaphylaxis, pulmonary embolism, retinal artery thrombosis, infection.</td>
</tr>
<tr>
<td></td>
<td>Splenectomy</td>
<td></td>
<td>Initial response rate above 80%,</td>
<td>Hemorrhage, peripancreatic hematoma,</td>
</tr>
<tr>
<td>TPO-RAs</td>
<td>Romiplostim doses 1-10 μg/kg</td>
<td>Initial response rate above 80%, sustained response with continual administration of the drug.</td>
<td>Headaches, fatigue, epistaxis, arthralgia and contusion, increased bone marrow reticulin, worsening thrombocytopenia upon discontinuation, thrombosis.</td>
<td>approximately two-thirds achieve a lasting response.</td>
</tr>
</tbody>
</table>
higher risk of bleeding and therefore most should be treated despite of bleeding symptoms (Provan et al., 2010; Thienelt and Calverley, 2009).

Fist-line treatments for ITP include steroids, IV anti-D and IVIg (Provan et al., 2010). Steroids therapies are a conventional and affordable first-line treatment option but have systemic and sometimes severe side effects that greatly reduce the quality of life of patients while IV anti-D and IVIg are more expensive but better tolerated (Provan et al., 2010). Commonly prescribed steroids include prednisone, dexamethasone, and methylprednisolone. Prednisone is recommended to be administrated at 0.5-2 mg/kg/d until the platelet count increase is above 30-50×10⁹/L and then administered at tapered doses or tapered if there is no response after 4 weeks. Dexamethasone was recommended at 40 mg/day for 4 days for once or for 4 cycles at an interval of 2 weeks. In the literature, high dose methylprednisolone is usually prescribed at 30 mg/kg for 7 days to patients who had failed other first-line therapies. IV anti-D is recommended for RhD⁺ non-splenectomized patients with ITP without autoimmune hemolytic anemia at a dose between 50-75 μg/kg for 4-5 days. IVIg is recommended at 0.4 g/kg for 5 days or 1 g/kg for 1-2 days.

Second-line treatments are generally recommended for patients who fail one or more of the first-line therapies with the goal of achieving relatively safe platelet counts (Provan et al., 2010). Second-line treatment options include azathioprine, cyclosporin A, cyclophosphamide, danazol, dapsone, mycophenolate mofetil, rituximab, splenectomy, TPO-RAs and vinca alkaloids (Provan et al., 2010). Of these, splenectomy has shown the highest long-term response rate, however about 20% fail to respond and another 10% patients may relapse after an initial response. Therefore, splenectomy is not recommended to patients within 6 months from the initial diagnosis of ITP. Other commonly prescribed second-line treatments include rituximab and the TPO-RAs, eltrombopag and romiplostim, with clear advantages including low toxicity and improved quality of life (Provan et al., 2010).

Emergency treatments are recommended to patients ‘needing surgical procedures, at high risk of bleeding, or with active central nervous system (CNS), GI, or genitourinary bleeding’,
including switching among first-line treatments or a combination of them or together with platelet transfusions (Provan et al., 2010).

**Treatment of pediatric ITP**

A difference between adult and pediatric ITP is that most children undergo spontaneous remission whereas adults usually do not. Moreover, only 3% of the children with ITP have clinically significant bleeding symptoms. Therefore, for newly diagnosed patients whose platelet counts are above 20×10^9/L without serious bleedings, observation without intervention is recommended (Provan et al., 2010; Thienelt and Calverley, 2009). For patients with platelet counts below 20×10^9/L or with serious bleedings, IV anti-D, IVIg and prednisone are recommended as first-line treatments. For the management of life-threatening bleedings, platelet transfusions together with IVIg, IV anti-D, or prednisone were recommended (Provan et al., 2010; Thienelt and Calverley, 2009).

**Management of ITP in pregnancy**

Both newly diagnosed ITP during pregnancy and preexisting ITP present during pregnancy are usually investigated and managed differently than regular adult ITP. During the first two trimesters of pregnancy, interventions are not recommended unless necessary when maternal platelet counts are below 20×10^9/L or there is significant bleeding. In the last a few weeks of pregnancy, a platelet count of 50-75×10^9/L or higher was recommended for anesthesia. First-line treatments are similar as with adult ITP, however, low dose steroids are recommended to minimize side effects (Provan et al., 2010; Thienelt and Calverley, 2009). Evidence for second-line treatments are limited but combinations of first-line treatments and rituximab have been shown effective. Splenectomy was recommended only during the second trimester when necessary.

**1.3.5 Intravenous immunoglobulin (IVIg) in ITP**

Intravenous immunoglobulin (IVIg) is prepared from pooled plasma of thousands of blood donors and mainly contains polyvalent IgG antibodies (Lazarus and Crow, 2003). It was initially used to treat hypogammaglobulinemic diseases (Etzioni and Pollack, 1989) but now, it has
become a first-line treatment for the management of ITP as well as other autoimmune diseases (Etzioni and Pollack, 1989; Lazarus and Crow, 2003).

In 1981 Imbach et al first reported increased platelet counts in pediatric patients with ITP after IVIg infusion in a pilot study (Imbach et al., 1981). After the initial success, Imbach and collaborators recruited more patients for IVIg therapy and showed that more than eighty percent of patients responded to IVIg, with a mean response time of 9 days (Imbach et al., 1985). Subsequently, IVIg was found effective in upregulating the platelet counts of adults with ITP with comparable initial response rates (Bussel et al., 1983; Fehr et al., 1982; Newland et al., 1983; Provan et al., 2010). However the treatment free response rate 4-6 weeks after the IVIg treatment was below 50% (Bussel et al., 1983; Fehr et al., 1982; Newland et al., 1983; Provan et al., 2010). Adverse events of IVIg therapy mostly are benign (Provan et al., 2010; Ryan et al., 1996). Chills and headaches are the most common, while other symptoms may occur including ‘transient neutropenia, renal insufficiency, aseptic meningitis, thrombosis, flushing, fever’, ‘fatigue, nausea, diarrhea, blood pressure changes and tachycardia’ (Provan et al., 2010; Ryan et al., 1996).

Possible mechanisms of action of IVIg have been investigated since its early application in ITP. In the study by Fehr et al in 1982, administration of autologous $^{99m}$Tc-labeled anti-Rh(D) IgG antibody opsonized RBCs in addition to IVIg showed prolonged RBC clearance time, suggesting that IVIg inhibited platelet clearance by macrophages (Fehr et al., 1982). Increased hemolysis determined by haptoglobin level after IVIg therapy supported this theory (Salama et al., 1983). Studies comparing intact immunoglobulins and Fc-depleted F(ab’)$_2$ fragments showed that the Fc portion of immunoglobulins is needed for the function of IVIg (Burdach et al., 1986; Crow et al., 2001; Tovo et al., 1984). These studies clearly indicate that at least one mechanism for the action of IVIg is through an inhibition of FcR-mediated platelet clearance by macrophages (Burdach et al., 1986; Crow et al., 2001; Tovo et al., 1984).

As mentioned in session 1.3.2.8, Clarkson et al showed that the blockade of FcγRII and FcγRIIIA receptors ameliorated ITP, indicating a major role of these two receptors in the clearance of platelets in ITP (Clarkson et al., 1986). Using an anti-platelet monoclonal antibody induced transient thrombocytopenia (passive ITP) mouse model in FcγRI, FcγRIIB and FcγRIII
deficient mice, Samuelsson et al showed that IVIg downregulated the platelet clearance through inhibition of FcγRIII in association with upregulation of the inhibitory receptor FcγRIIB (Samuelsson et al., 2001). This was concurrently confirmed in the studies by Crow and Teeling (Crow et al., 2003a; Teeling et al., 2001). Furthermore, Siragam et al found that IVIg specifically downregulated FcγRIIIA on CD11c⁺ DCs independent of FcγRIIIB (Siragam et al., 2006).

In addition to the modulation of FcR mediated platelet destruction, IVIg also ameliorates ITP through other mechanisms. For example, in 1985, Delfraissy et al proposed a mechanism of IVIg through the improvement of suppressor cells, based on their findings of normalized proliferation of the PBMCs from IVIg responders upon Concanavalin A stimulation in vitro (Delfraissy et al., 1985). Kaneko et al in Ravetch group associated the anti-inflammatory role of IVIg with the sialylation of its Fc fragments (Kaneko et al., 2006). In the same year, Leytin et al examined the effect of IVIg on platelets and found significantly decreased platelet apoptosis after IVIg treatment (Leytin et al., 2006). Our previous work a few years ago showed increased suppressive Tregs in IVIg treated ITP mice (Aslam et al., 2012). More recently, Leontyev et al examined the impact of IVIg therapy on cytokine production in antibody-mediated passive ITP mice (Leontyev et al., 2014). They found that in passively-induced ITP mice of either the BALB/c or C57BL/6J genetic backgrounds, IVIg stimulated the production of IL-4, IL-10, IL-11, IL-23, granulocyte-macrophage–colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and tumor necrosis factor α (TNF-α). Increased MCP-3 and chemokine (C-C motif) ligand 5 (CCL5, also known as RANTES, an abbreviation of ‘released upon activation, normal T cell expressed and secreted’) were also increased by IVIg treatment, but only BALB/c strain mice (Leontyev et al., 2014). Taken together, it is clear that the mechanism of action of IVIg is complicated and still not fully elucidated.

1.3.6 Platelet transfusions in ITP

Since the 1950s, allogeneic platelet transfusions have been used clinically to rescue platelet counts in thrombocytopenic disorders such as the severe thrombocytopenia secondary to leukemia (Han et al., 1966; Hirsch and Gardner, 1952; Slichter, 1980). However, in the case of
ITP, historically, allogeneic platelet transfusions were not recommended. In the report by Sprague et al in 1952, entitled: “Platelet transfusions and the pathogenesis of idiopathic thrombocytopenic purpura”, it was suspected that platelets transfused into patients with ITP ‘disappear within several hours from the circulation’ as suggested by the quick drop of platelet counts after platelet transfusions, whereas in patients with aplastic anemia, transfused platelets could survive for 4-6 days (Sprague et al., 1952). Therefore, although substantial evidence was lacking, platelet transfusions were subsequently considered ‘unnecessary and undesirable, considering the risks of hepatitis and the lack of effectiveness’ (Ahn and Harrington, 1977). There were also assumptions that patients with ITP would not require platelet transfusions ‘because the compensatory marrow response is associated with a reduction in megakaryocyte maturation time and release of young hyperfunctional platelets’ (Slichter, 1980). Although platelet production by megakaryocytes has clearly been shown to be impaired in ITP (Chang et al., 2003; Li et al., 2007) and to date, no supporting evidence exists that the platelets from patients with ITP are actually ‘hyperfunctional’, the concept that platelet transfusions should be avoided for the management of ITP is still prevailing (Provan et al., 2010; Thienelt and Calverley, 2009).

Contrary to the dogma that platelet transfusions should be avoided in ITP, there is evidence that supports the effectiveness of platelet transfusions in the immediate control of active bleeding. In 1986, a retrospective evaluation of 11 patients with ITP showed efficient control of active bleeding after platelet transfusions with approximately half of the transfusions increasing immediate platelet counts and 16% of the transfusions inducing next-day platelet count increments (Carr et al., 1986). This study not only challenged the dogma that platelet transfusions were not effective in ITP, but suggested a therapeutic benefit of platelet transfusions in ITP. Furthermore, Salama et al showed that massive platelet transfusions given as the only treatment for 10 cases of refractory ITP increased the platelet counts and stopped active bleeding in all patients without serious side effects (Salama et al., 2008). In another study, Spahr et al demonstrated that platelet transfusions administered concurrently with IVIg could significantly raise the platelet counts to 50×10⁹/L (averaged below 10×10⁹/L before treatment) in more than half of patients with ITP (Spahr and Rodgers, 2008). Similarly, in the IWG guideline, platelet transfusions in combination with other first-line treatments, including IVIg,
were recommended for the management of life-threatening bleeding or preparation for surgery (Provan et al., 2010). Concurrently, Thachil et al also recommended that in ITP and other immune mediated thrombocytopenic disorders, platelet transfusions ‘should not be withheld if a safe platelet count is preferable’ (Thachil, 2010). Overall, to date, the clinical evidence suggests that platelet transfusions may in fact be effective in ITP and even more importantly, could control active bleeding within short time periods. However, since there is limited evidence in the literature, future evaluations of the effectiveness and safety of allogeneic platelet transfusions in the management of ITP are still needed.

1.3.7 Rituximab and B cell depletion therapy

Rituximab is a purified monoclonal chimeric antibody derived from fusing the V region of a mouse anti-human CD20 antibody to a human IgG1 constant segment (Reff et al., 1994). Rituximab targets CD20 on B cells and induces B cell depletion in vivo. It was initially licensed as an immunotherapy for B cell lymphoma and now is also used as a treatment for several autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, autoimmune haemolytic anemia, multiple sclerosis, diabetes and ITP (Brodie et al., 2008; Cohen et al., 2006; Cross et al., 2006; Gurcan et al., 2009; Hu et al., 2007; Leandro et al., 2002; Lund and Randall, 2010; Pescovitz et al., 2009; Stasi et al., 2001). Several mechanisms of how rituximab causes B cell depletion in vivo have been proposed including Fc-receptor mediated phagocytosis of antibody coated B cells, killing of B cells through antibody-dependent cellular cytotoxicity (ADCC), complement-dependent lysis of B cells or sensitization of B cells to apoptosis (Cardarelli et al., 2002; Golay et al., 2000; Gurcan et al., 2009; Lefebvre et al., 2006; Pedersen et al., 2002). The application of rituximab in ITP management was first reported by Stasi et al in 2001 (Stasi et al., 2001). In their study, rituximab was given at a dose of 375 mg/m² once weekly for 4 weeks to 25 patients with chronic ITP who had failed at least 2 different therapies. More than 50% of the patients responded to rituximab, and 20% of them had a complete response and 28% still had treatment-free remission after 6 months. The therapy was well tolerated in all patients with only minor side effects that were mostly fevers and chills. With larger scale patient recruitment and longer term follow up, the overall response of rituximab in adult ITP has been reported to be approximately 60%, with a 2-year response rate of 40% and a 5-year remission rate of 20% (Khellaf et al., 2014; Patel et al., 2012).
Rituximab in combination with other therapies, such as corticosteroids or thrombopoietin receptor agonists, have also shown synergistic effects (Gudbrandsdottir et al., 2013; Zhou et al., 2015). For example, in a randomized clinical trial, rituximab and dexamethasone combined therapy showed significantly higher response rates than dexamethasone monotherapy after 6 months and longer time to relapse (Gudbrandsdottir et al., 2013). Moreover, compared with classical corticosteroid therapy or splenectomy, rituximab is well-tolerated with fewer side effects. Of the 248 rituximab treated ITP patients in the study by Khellaf et al, only 19% of patients had adverse events, most of which were minor except 3 events (approximately 1%) that required interruption of the treatment (Khellaf et al., 2014). Therefore with the consideration of the balance between efficacy and side effects, rituximab is a preferred treatment for the management of ITP.

Efforts have been made to understand the therapeutic mechanisms of rituximab since its initial success in ITP management (Stasi et al., 2001). It was suspected that rituximab could rescue the antibody-mediated ITP through removal of B cells (Stasi et al., 2001). Although it was confirmed in subsequent studies that the antibody-platelet antibody titers decreased after remission in some patients, it was also found that the antibody titer may be unchanged in patients under remission as well (Cooper et al., 2012). This suggests that there must be some other antibody-independent mechanism(s) of rituximab therapy. The effectiveness of rituximab in some antibody negative patients also indicates alternative explanations for the in vivo effect of rituximab (Cooper et al., 2012). In 2007, Stasi et al reported normalized Th1/Th2 (IFN-γ+/IL-4+ of CD4+ T cells) and Tc1/Tc2 (IFN-γ+/IL-4+ of CD8+ T cells) ratios in the peripheral blood of rituximab responders at 3 and 6 months after treatment but not in patients with active ITP nor in rituximab non-responders, suggesting an immunomodulatory effect of rituximab on both CD4+ and CD8+ T cell responses (Stasi et al., 2007). Nazi et al also showed that the number of IFN-γ+–producing T cells upon exogenous antigen stimulation was decreased in patients with ITP who received rituximab administration compared with patients without rituximab treatment (Nazi et al., 2013). Subsequently, further examinations of the CD4+ T cells revealed restored Treg number and function in the peripheral blood in rituximab responders (Stasi et al., 2008). Of interest, the number of Tregs remained low in the spleen of rituximab non-responders (Audia et
al., 2011). However, it remains unclear how rituximab regulates T cell-mediated immune responses in ITP.

1.3.8 Animal models of ITP

Animal models of human diseases provide a valuable tool to facilitate investigations into the pathogenesis of disease and to evaluate therapeutic reagents and procedures prior to clinical trials. Several animal models of ITP have contributed to our understanding of ITP and are still of relevance today in human medicine as reviewed by Semple (Semple, 2010). Animal models of ITP can be divided into three categories: passive ITP (anti-platelet serum/antibody induced), secondary ITP (drug/infections/other diseases induced) and active ITP (anti-platelet immune cell-induced).

Passive ITP

Passive ITP models were the earliest developed and are still the most commonly used animal models of ITP. Since the 1950s, passive ITP models induced by anti-platelet sera in guinea pigs, swine, rodents, and rabbits etc have been used for the understanding of the pathology and treatments of thrombocytopenia (Semple, 2010). For example, in 1960, using a guinea pig passive ITP model induced by anti-platelet serum, Rossolini et al showed that cortisone was effective in ameliorating thrombocytopenia. A few years later, cortisone was further found effective in a neonatal thrombocytopenia model as well (Chieffi et al., 1964; Semple, 2010). In the 1970s and 1980s, rodent passive ITP models were used for the study of TPO and megakaryopoiesis during thrombocytopenia (Dassin et al., 1983; Kalmaz and McDonald, 1981; Nakeff and Roozendaal, 1975; Rolovic et al., 1970; Semple, 2010). These early models using immune animal sera laid the groundwork for understanding different mechanisms related to ITP. With the advent of monoclonal antibody technology, anti-platelet monoclonal antibodies are commonly used to induce thrombocytopenia today.

In 1981, the earliest anti-platelet monoclonal antibodies (mAb) were developed by McMichael and Newman respectively (McMichael et al., 1981; Newman et al., 1981). Since then, passive ITP models induced by monoclonal antibodies have been widely used. For example, the passive ITP model was used to improve our understanding of the therapeutic
effects of IVIg in ITP. In 1987, using the monoclonal antibody induced passive ITP rat model, Tsubakio et al. showed the IVIg as an effective therapy for ITP (Tsubakio et al., 1987). Later, the anti-platelet antibody-mediated passive ITP mouse model was commonly used in further studies of different FcRs in ITP and IVIg therapies by several groups, including the studies by Samuelsson et al. and Siragam et al. mentioned in session 1.3.4 (Crow et al., 2003a; Samuelsson et al., 2001; Siragam et al., 2006; Teeling et al., 2001). More recently, the passive ITP mouse model also helped to evaluate the role of Fc\(\gamma\)RIIB in ITP development. In Fc\(\gamma\)RIIB deficient mice, passively infused anti-platelet antibodies could still mediate thrombocytopenia and IVIg as well as anti-CD44 antibodies were found effective in certain strains of Fc\(\gamma\)RIIB deficient mice suggesting a dispensable role of the Fc\(\gamma\)RIIB in ITP (Crow et al., 2015; Leontyev et al., 2012). In addition, there are studies using splenectomized mice to help understand the role of IVIg in splenectomized patients (Leontyev et al., 2012; Schwab et al., 2012). Taken together, these studies using the passive ITP mouse model in genetically altered animals not only improved our understanding of both ITP and the in vivo effects of IVIg on the immune system.

In the last decade, the passive ITP model has also been utilized for the study of the immune functions of platelets and their interaction with other immune cells as well. In 2004, our group showed that LPS could bind to TLR4 on platelets and induce increased platelet destruction and development of thrombocytopenia in vivo (Aslam et al., 2006). The LPS also significantly enhanced FcR-mediated phagocytosis of anti-platelet IgG opsonized platelets in vitro, suggesting that signaling through TLRs on platelets may exacerbate ITP (Semple et al., 2007).

Passive ITP models also allowed for comparisons of different monoclonal antibodies in the pathogenesis of ITP. For instance, it has been shown that anti-GPIb antibodies but not anti-GPIIbIIIa antibodies are capable of inducing Fc-independent desialylation of sialic acid on platelets and this leads to increased platelet destruction through the Ashwell-Morell receptors and development of thrombocytopenia in vivo (Li et al., 2014; Li et al., 2015b). However, there are still controversies and future investigations are still needed to confirm the role of anti-GPIb antibodies in platelet desialylation and ITP development. We also compared different anti-GPIb and anti-GPIIbIIIa antibodies and their effects on megakaryocytes in vivo and this will be discussed in Chapter 2.
Studies using CD47 deficient mouse models also revealed a potential novel mechanism in ITP. It has been shown that CD47 deficient platelets from CD47+/− or CD47−/− mice induced increased phagocytosis by macrophages in vitro as well as rapid platelet clearance in vivo (Olsson et al., 2005). The CD47 deficient platelets also exacerbated the antibody-mediated passive ITP in mice (Olsson et al., 2005). Moreover, Guo et al have shown that a bacteria strain Escherichia coli O157:H7 could down regulate CD47 expression on platelets and upregulate the phagocytosis of platelets by macrophages to induce thrombocytopenia in mice (Guo et al., 2009).

**Secondary ITP Models**

In the literature, there are a broad variety of different secondary ITP animal models which are relevant to the study of secondary ITP in humans. For example, quinine induced ITP was probably the earliest secondary ITP animal model developed. In 1964, Wakisaka et al studied quinidine induced ITP in dogs (Semple, 2010; Wakisaka et al., 1964) and in 1977, Kekomaki et al studied aspirin induced ITP in rabbits. In both cases, it was the binding of the drug to the animal’s platelets that triggered antibody production against platelet-drug complexes and the ultimate destruction of platelets (Kekomaki et al., 1977). Other drugs that may cause secondary ITP in mice include sulphonamide, phenobarbitol, pantethine, gold, and heparin.

Infection-induced secondary ITP in animals was first reported by Musaji et al (Musaji et al., 2004). The authors showed exacerbated thrombocytopenia when mice with passive ITP were infected with mouse viruses (Musaji et al., 2004). Subsequently, Tremblay et al confirmed Semple et al’s in vitro results by showing that LPS could exacerbate passive ITP in mice (Tremblay et al., 2007). These latter models may be a useful tool to improve our understanding of the immunological functions of platelets and their role in regulating the immune system.

**Active ITP**

Active murine ITP was developed as an adoptive transfer model to induce both antibody- and T cell-mediated ITP by our group (Chow et al., 2010). In this model, GPIIIa (CD61, β3 integrin) knockout (KO) mice were immunized by multiple wildtype (WT, CD61+) platelet transfusions and when immune, they are sacrificed and their splenocytes adoptively transferred into mice with severe combined immunodeficiency (SCID) to induce ITP (Chow 2010). By
removal of either CD8+ T cells or B cells from the splenocytes before adoptive transfer, antibody-mediated or CD8+ T cell-mediated ITP respectively, could be induced. It was found that while IVIg treatment was effective in raising platelet counts in antibody-mediated ITP, it did not affect the thrombocytopenia mediated by CD8+ T cells (Chow et al., 2010). Moreover, subsequent examination of the thymus from ITP SCID mice revealed increased retention of Tregs in the thymus which may help explain the Treg deficiency in human ITP (Aslam et al., 2012). In this thesis, I examined two ITP therapies in our active murine model: allogeneic platelet transfusion therapy and B cell depletion therapy (Chapters 3 and 4). My work not only showed consistent findings as in human patients but also revealed novel mechanisms that may help to explain certain phenomena in human ITP.

In summary, animal models of ITP have improved our understanding of the anti-platelet immune responses in ITP and the actions of therapeutic reagents. Furthermore, it appears that the active ITP model could be helpful for the preclinical evaluation of novel therapies for patients with ITP.
1.4 Rationale, Hypothesis and Specific Aims

1.4.1 Rationale

Immune thrombocytopenia (ITP) is a heterogeneous autoimmune disease where both B cell and CD8$^{+}$ T cell abnormalities are involved. In the pathogenesis of the disease, the relative contributions of B cells and CD8$^{+}$ T cells are difficult to distinguish from each other in patients. In addition, the evaluation of how different therapies rescue the disease under these different B or T cell circumstances is even more complicated. Moreover, megakaryocytes are not routinely examined in ITP despite their critical role for platelet production and therefore our knowledge of megakaryocytes before and after treatments is still limited. Animal models of ITP thus are valuable tools that will allow us to differentiate the antibody-mediated and CD8$^{+}$ T cell-mediated ITP and examine the mechanisms of treatments as well as their effect on the megakaryocytes and platelet production.

Although anti-platelet antibodies with different antigen specificities and isotypes have been widely reported in human ITP, the antibody effects on megakaryocytes and thrombopoiesis have not been well characterized. In addition, IVIg is a first-line treatment for ITP and has been found effective in reducing anti-platelet antibody-mediated peripheral platelet destruction but its effect on megakaryocytes in vivo is not clear. Thus, characterization of the effects of different antibodies and IVIg on megakaryocytes may improve our understanding of the disease and the IVIg therapy.

Allogeneic platelet transfusion therapy is another treatment option under emergent situations for the management of ITP but its application in ITP is still controversial. Historically, its use was frowned upon for the management of ITP, however, limited clinical evidence has emerged that suggests that transfusions may be effective in raising platelet counts and controlling bleeding symptoms in ITP. Investigations on allogeneic platelet transfusions in either antibody-mediated or T cell-mediated ITP using our active ITP mouse model may provide helpful information for clinical practice.

B cell depletion in vivo using rituximab is also a common treatment for the management of ITP. The unchanged antibody titer in rituximab responders and the effectiveness of rituximab in
certain anti-platelet antibody negative patients suggest that antibody-independent therapeutic effects of rituximab exist but their mechanism(s) is unknown. Examination of the effect of B cell depletion on CD8⁺ T cell-mediated ITP in our mouse model may uncover novel mechanisms of the treatment and improve our understanding of the interaction between B cells and T cells in ITP.

1.4.2 Hypothesis

1) Antibodies of different specificities and isotypes induce thrombocytopenia in vivo but do they interfere with megakaryocytes in the bone marrow?

2) IVIg can rescue antibody-mediated thrombocytopenia but does it have effects on megakaryocytes?

3) Allogeneic platelet transfusions have immunomodulatory effects and may suppress CD8⁺ T cell-mediated ITP.

4) Does B cell depletion therapy affect suppress CD8⁺ T cell-mediated ITP?

1.4.3 Specific aims

1) Compare the effects of different anti-platelet antibodies/sera on megakaryocytes and thrombopoiesis and evaluate the efficacy of IVIg on megakaryocytes in antibody-mediated passive ITP.

2) Examine the effect of allogeneic platelet transfusions on CTL-mediated active ITP, including their effects on megakaryocytes and analyze the CTL responses against platelet CD61 versus MHC antigens in active ITP.

3) Evaluate the efficacy of anti-mouse CD20 IgG2a antibody on B cell depletion in vivo and investigate the effects of the anti-CD20 antibody on CTL-mediated ITP.
Chapter 2

Thrombocytopenia And Megakaryocyte Counts Are Differentially Controlled By The Specificity Of Anti-Platelet Antibodies In A Murine Model Of Passive Immune Thrombocytopenia (ITP)

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*L.G and *R.A. contributed equally to the paper.
2.1 Abstract

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by increased peripheral immune platelet destruction and megakaryocyte defects in the bone marrow. Although ITP was originally thought to be primarily due to antibody-mediated autoimmunity, it is now clear that T cells can also play a significant role in the disease. However, the exact interplay between platelet destruction, megakaryocyte dysfunction, and the elements of both humoral and cell-mediated immunity in ITP remain incompletely understood. While most studies have focused on immune platelet destruction in the spleen, an additional possibility is that the anti-platelet antibodies also have effects on bone marrow megakaryocytes. To address this, we utilized an in vivo passive ITP mouse model, where BALB/c mice were administered various anti-GPIIb, GPIIIa or GPIb monoclonal antibodies or anti-platelet sera, followed by platelet counts and bone marrow megakaryocytes enumeration. Our results show that after 24 hours, all the different anti-platelet antibodies/sera induced significant thrombocytopenia in recipient mice. IVIg raised the platelet counts in all the thrombocytopenic mice except mice in NIT-E antibody (an anti-GPIb antibody) induced group. Compared with naïve control mice, however, histological examination of the bone marrow revealed that only two antibody preparations (mouse-anti-mouse β3 sera and an anti-GPIIb monoclonal antibody, MWReg30) increased bone marrow megakaryocyte counts, and IVIg only partially normalized the number of megakaryocytes in MWReg30 group. Our study suggests that most of the anti-platelet antibodies may not have direct effect on megakaryocytes in vivo, and the bone marrow effects observed in ITP may involve T cell responses. Moreover, IVIg may not directly affect thrombopoiesis.
2.2 Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disease characterized by an isolated thrombocytopenia without any other hematological abnormalities except a possible anemia due to blood loss (Rodeghiero et al., 2009). The pathogenesis of ITP is complex both at the level of thrombohaemostasis and immune system regulations. For example, the thrombocytopenia of ITP is due to at least two major mechanisms: either increased peripheral platelet destruction in the spleen or reduced platelet production from bone marrow (Ballem et al., 1987; Cines et al., 2014; Shulman et al., 1965). The mechanism of peripheral platelet destruction in ITP has been studied extensively and is thought to be primarily due to increased FcR-dependent phagocytosis of opsonized platelets by splenic macrophages (Cines et al., 2014; Harrington et al., 1951; Shulman et al., 1965). This immune platelet destruction is associated with a vast array of peripheral and splenic B cell and T cell defects involving, for example, cytokine defects, Th1-skewness, regulatory T cell abnormalities and loss of tolerance (Cines et al., 2014; McKenzie et al., 2013).

Less is known, however, about the immune mechanism(s) of reduced platelet production in ITP although there are some early and recent clues (Dameshek and Miller, 1946; Houwerzijl et al., 2004). Probably the first report related to reduced platelet production in ITP was in 1946 when Dameshek and Miller examined the bone marrow of patients with ITP and found increased numbers of megakaryoblasts along with elevated numbers of degenerated megakaryocytes and decreases in the number of mature megakaryocytes shedding platelets (Dameshek and Miller, 1946). Houwerzijl et al. confirmed these findings by studying the fine ultrastructural characteristics of bone marrow megakaryocytes from patients with ITP using EM (Houwerzijl et al., 2004). Of interest, they observed apoptotic morphological abnormalities of megakaryocytes such as cytoplasmic vacuolization and chromatin condensation and this was in consistency with increased caspase-3 expression in the megakaryocytes (Houwerzijl et al., 2004). The immune nature of the megakaryocyte abnormalities was first shown by two groups that both demonstrated reduced in vitro megakaryocyte growth upon addition of anti-platelet
autoantibodies with various specificities e.g. anti-GPIb and anti-GPIIbIIIa (Chang et al., 2003; McMillan et al., 2004).

More recently, it has become evident from murine models that cytotoxic T cells may also play a significant role in megakaryocyte destruction in ITP (Chow et al., 2010; Guo et al., 2014). However, the exact interplay between megakaryocyte dysfunction, platelet production and the elements of both humoral and cell mediated immune systems remain ambiguous. To better address how anti-platelet antibodies solely affect megakaryocyte numbers and morphology in vivo, we utilize a well-characterized murine model of passive ITP and demonstrate that while most antibodies mediate thrombocytopenia, not all are associated with in vivo megakaryocyte effects. The results suggest that although megakaryocytes and platelets share similar epitopes that could be recognized by anti-platelet antibodies, most antibodies do not have direct effects on megakaryocytes in vivo. If so, the megakaryocyte abnormalities in patients with ITP may occur due to other immune mechanisms such as T cell-mediated immune responses.
Table 2.1 Details of polyclonal (serum) and monoclonal antibodies used for ITP induction

**TABLE 1. Details of polyclonal (serum) and monoclonal antibodies used for ITP induction**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sera/mAb</th>
<th>Specificity</th>
<th>Host</th>
<th>Isotype</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-β3</td>
<td>sera</td>
<td>β3 integrin</td>
<td>mouse</td>
<td>IgG</td>
<td>200 – 250μL</td>
</tr>
<tr>
<td>RAMS</td>
<td>sera</td>
<td>mouse platelet</td>
<td>rabbit</td>
<td>IgG</td>
<td>5μL</td>
</tr>
<tr>
<td>MWReg30</td>
<td>mAb</td>
<td>αIIb integrin</td>
<td>rat</td>
<td>IgG1</td>
<td>2-4μg</td>
</tr>
<tr>
<td>2C9.G2</td>
<td>mAb</td>
<td>β3 integrin</td>
<td>Armenian Hamster</td>
<td>IgG1</td>
<td>10μg</td>
</tr>
<tr>
<td>NIT-B</td>
<td>mAb</td>
<td>glycoprotein Ib</td>
<td>mouse</td>
<td>IgG2a</td>
<td>1μg</td>
</tr>
<tr>
<td>NIT-E</td>
<td>mAb</td>
<td>glycoprotein Ib</td>
<td>mouse</td>
<td>IgG2b</td>
<td>1μg</td>
</tr>
<tr>
<td>NIT-G</td>
<td>mAb</td>
<td>glycoprotein Ib</td>
<td>mouse</td>
<td>IgG1</td>
<td>1μg</td>
</tr>
<tr>
<td>NIT-H1</td>
<td>mAb</td>
<td>glycoprotein Ib</td>
<td>mouse</td>
<td>IgG1</td>
<td>1μg</td>
</tr>
</tbody>
</table>

Anti-β3=mouse anti-mouse β3 serum; RAMS=rabbit anti-mouse platelet serum.
2.3 Materials and Methods

2.3.1 Mice

BALB/c mice (H-2\textsuperscript{d}, Charles River Laboratories, Montreal, QC, Canada) were used as antibody recipients. BALB/c CD61 (GPIIIa, β3 integrin) knockout (KO) mice were supplied by Dr. Heyu Ni and used as a source of mouse anti-mouse β3 sera. All mice were 8-12 weeks of age and all studies were approved by the St. Michael’s Hospital Animal Care Committee.

2.3.2 Anti-platelet antibodies and Passive ITP

Rabbit-anti-mouse platelet serum (RAMS, Cedarlane Laboratories, Mississauga, ON) was obtained from Dr. Alan Lazarus (St. Michael’s Hospital). Monoclonal antibodies (mAbs) MWReg30 and 2C9.G2 were obtained from BD Biosciences (Cat No. 553847, 553344). Monoclonal antibodies NIT-B, NIT-E, NIT-G, and NIT-H1 were obtained from Dr. Heyu Ni (St. Michael’s Hospital). Mouse-anti-mouse β3 sera (i.e. anti-CD61, or anti-GPIIIa) was obtained from β3 KO mice immunized with syngeneic wildtype (WT) platelets which are positive for β3 as previously described (Chow et al., 2010; Guo et al., 2014). The antigen specificities, isotypes as well as the doses used for ITP induction of all the sera and antibodies are listed in Table 2.1. Doses were determined according to literature and our experience in inducing comparable thrombocytopenia in all mice (Semple, 2010). PBS was used as a sham control injection. To passively induce ITP, all sera and monoclonal antibodies were administered by an intraperitoneal (ip) injection. For intravenous immunoglobulin (IVIg) treatment of ITP, IVIg was administered ip at 2 g/kg, 5 min prior to the antibody injection.

2.3.3 Platelet counts

Mice were bled via the saphenous vein and 10 μL peripheral blood was collected into PBS with 10% CPDA. Platelet counts were measured using a Beckman Coulter Counter-LH750 hematology analyzer as previously described (Chow et al., 2010; Guo et al., 2014).

2.3.4 Bone marrow histology and megakaryocyte count
Mice were sacrificed 24 h after monoclonal antibody or anti-platelet serum injection. Femurs were collected, dissected of muscle tissue and the epiphyses were cut off and the bone shafts were placed in fixative (B+ fixative; BBC Biochemicals) for 12 hours. The bones were further decalcified with 10% nitric acid for 2 hours and then processed and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). All pictures were taken using the CellSens Standard software under a 40× objective lens (Olympus BX50), with the numerical aperture of objective lens 0.75. Images of 10 random fields (450μm×320μm/field) throughout each slide were taken from every mouse (Guo et al., 2014). The number of megakaryocytes on each image was recorded for the calculation of average value.

2.3.4 Statistical analysis

Statistical analyses were performed using a computer software Prism (GraphPad Software, Version 6.0, San Diego, CA). Data are presented as mean ± standard deviation (SD). One-way ANOVA with Tukey’s post-hoc test and t test were used for data analysis, and p values of less than 0.05 were regarded as significant.
2.4 Results

2.4.1 The severity of thrombocytopenia varies depending on the anti-platelet antibody used

Figure 2.1 A shows the platelet counts of BALB/c mice 24 h after injection of either PBS, or the indicated anti-platelet sera or mAbs. Compared with the PBS control, the anti-β3 sera (i.e. anti-GPIIIa or anti-CD61 sera) and RAMS induced the most severe (p<0.0001) thrombocytopenia in recipient mice by 24 h post-injection. All the mAbs also induced significant thrombocytopenia in the BALB/c recipient mice (Figure 2.1 A). The severity of the thrombocytopenia in these mice was comparable to anti-β3 sera and RAMS induced, except NIT-E group. The thrombocytopenia induced by NIT-E was more moderate compared with RAMS or MWReg30 (Figure 2.1 A), although all the NIT-B, NIT-E, NIT-G and NIT-H1 antibodies were of the same host and similar specificity (anti-GPIIb). Treatment of the recipient mice with 2 g/kg IVIg significantly alleviated the thrombocytopenia in the mice except for the mice treated with mAb NIT-E (Figure 2.1 B).

2.4.2 Anti-β3 sera and mAb MWReg30 significantly increased the number of megakaryocytes in the bone marrow

Examination of the megakaryocyte counts in the bone marrow also showed variable effects by injection of the anti-platelet reagents. For example, although all the sera and mAbs induced significant thrombocytopenia, only the anti-β3 sera and mAb MWReg30 significantly increased the number of megakaryocytes as compared to controls (Figure 2.2 A). In addition, these two anti-platelet reagents caused significant morphological abnormalities, such as pyknotic nuclei and cytoplasmic blebbing (Figure 2.3). Among all groups, MWReg30 treated mice showed the highest megakaryocyte count which was significantly higher compared with all of the other groups. Megakaryocyte counts in the anti-β3 group were higher than the 2C9.G2, NIT-G and NIT-H1 treated groups (Figure 2.2 A). In contrast, the other groups did not show any significant impact on megakaryocytes. With IVIg treatment, megakaryocyte counts were only modified in
the MWReg30 group (Figure 2.2 B). There were no significant changes in the megakaryocyte counts of the rest of the groups after IVIg treatment (Figure 2.2 B). Representative images from control, MWReg30 treated and MWReg30 plus IVIg treatment groups are shown in Figure 2.3.
Figure 2.1 Platelet counts induced by the indicated anti-platelet antibodies and IVIg effects.

(A) From left to right, BALB/c mice received: PBS, mouse-anti-mouse β3 sera (anti-β3), rabbit-anti-mouse platelet sera (RAMS), and MWReg 30, 2C9.G2, NIT-B, NIT-E, NIT-G, NIT-H1 mAbs respectively. Comparisons among all groups were analyzed using one-way ANOVA with a Tukey’s post-hoc test (N=3-12, *P<0.05, ****P<0.0001). All the * labeled indicate P values when compared to Control. (B) Comparison of platelet counts between IVIg treated (spotted bars) and untreated mice (filled bars) that received PBS or indicated anti-platelet sera or mAbs. Student’s t test was used for the comparisons of each pair (N=3-12, *P<0.05, **P<0.01, NS=non-significant).
Figure 2.2 MWReg30 significantly increased the megakaryocyte counts.

(A) The megakaryocyte counts in the bone marrow of mice treated with indicated sera or mAbs. Comparisons among all groups were analyzed using one-way ANOVA with a Tukey’s post-hoc test (N=3-12, *P<0.05, **P<0.01, ****P<0.0001). (B) Comparison of megakaryocyte counts between IVIg treated (spotted bars) and untreated mice (filled bars) that received PBS or indicated anti-platelet sera or mAbs. Student’s t test was used for the comparisons of each pair (N=3-12, *P<0.05). The megakaryocyte counts of each mouse were calculated from the average value of 10 random fields.
Figure 2.3 Megakaryocyte morphology induced by MReg30 and IVIg effects.

Representative H&E stained bone marrows from BALB/c received
(A) PBS
(B) MWReg30
(C) IVIg and MWReg30 respectively.
Pictures were taken at 40× magnification with a 0.75 numerical aperture of the objective lens for all panels. The arrows point to megakaryocytes.
2.5 Discussion

In the current study, the effects of different anti-platelet antibodies on platelet and megakaryocyte counts were examined independently of T cells in a murine passive ITP model. While all the anti-platelet sera and monoclonal antibodies (mAbs) induced variable degrees of significant thrombocytopenia in BALB/c mice within 24 h of administration, only two anti-platelet reagents caused significant elevations and abnormal morphology in bone marrow megakaryocytes. These results suggest that antibodies of different anti-platelet specificity and isotypes have different biological effects in vivo. Most antibodies did not show any direct effect on megakaryocytes in our current study. In addition, while IVIg administration successfully alleviated the thrombocytopenia induced by the anti-platelet antibodies, its effect on megakaryocyte counts and morphology was limited within 24 hours.

Megakaryocytes are a specialized cell population in the bone marrow whose primary role is to produce platelets and they develop from CD34+CD41+ progenitor cells to become lineage restricted with the expression of CD61 (also known as integrin β3, GPIIIa) and CD41 (integrin αIIb, or GPIIb) (Battinelli et al., 2007; Italiano Jr. and Hartwig, 2013; Machlus and Italiano, 2013). Their development can be divided into 3 different stages: megakaryoblasts, promegakaryocytes, and mature megakaryocytes (Dameshek and Miller, 1946; Italiano Jr. and Hartwig, 2013; Long and Williams, 1981) and their maturation and proliferation are critically dependent on the megakaryocyte growth factor thrombopoietin (TPO) (Gurney et al., 1994). Mature megakaryocytes eventually release proplatelets into the bloodstream which become circulating platelets (Behnke, 1969; Italiano et al., 1999; Junt et al., 2007; Patel et al., 2005). In ITP, although antibody-mediated peripheral destruction of platelets was the earliest manifestation discovered (Harrington et al., 1951), it is now known that platelet production defects also occur and lead to thrombocytopenia but little is known of how reduced platelet production in the bone marrow is mediated.

Both anti-platelet antibodies and CD8+ T cells from patients with ITP have been studied in vitro for their effects on megakaryocyte maturation and platelet production. In 2003, Chang et al showed that plasma from ITP patients as well as purified anti-platelet monoclonal antibodies could inhibit the differentiation of megakaryocytes from umbilical cord blood mononuclear cells
in vitro (Chang et al., 2003). McMillan et al had consistent findings of decreased yield of megakaryocytes from CD34+ progenitor cells when cultured with plasma from patients with ITP. They further showed that anti-platelet antibodies from patients with ITP could also inhibit the maturation of megakaryocytes as indicated by the decreased ploidy of megakaryocytes in vitro (McMillan et al., 2004). Subsequently, Balduini showed that anti-GPIIbIIla antibodies inhibited pro-platelet formation by megakaryocytes in vitro, suggesting an additional mechanism that contributes to the thrombocytopenia in ITP (Balduini et al., 2008). Recently, Liu et al also showed impaired megakaryocyte production induced by allogeneic anti-platelet antibodies (Liu et al., 2015). It was also shown that CD8+ T cells could significantly induce megakaryocyte abnormalities and impair platelet production by megakaryocytes both in human and mouse (Chow et al., 2010; Li et al., 2007). Despite these observations, to our knowledge, the in vivo effects of anti-platelet antibodies, independently of CD8+ T cells, on the megakaryocytes in ITP have not been studied.

Animal models provide a valuable tool to facilitate our understanding of the pathogenesis of ITP (Semple, 2010). Passive ITP is the most commonly used mouse ITP model and is generated by injecting anti-platelet sera or mAb into mice (Semple, 2010). For example, in 1981, RAMS was used to induce thrombocytopenia, for the study of changes in small acetylcholinesterase-positive cells in the bone marrow, which were recognized as megakaryocyte progenitor cells (Kalmaz and McDonald, 1981). The earliest anti-platelet monoclonal antibodies were probably developed in 1981 by McMichael and Newman independently (McMichael et al., 1981; Newman et al., 1981) and in 1992, one of the first anti-murine platelet mAbs, MWReg30 was developed by Burstein et al and has been widely used to induce passive ITP (Burstein et al., 1992). In the current study, all the anti-platelet sera and mAbs induced significant thrombocytopenia within one day, irrespective of being from different hosts or IgG isotypes (Figure 2.1 and Table 2.1). This is consistent with our previous studies and others (Crow et al., 2003b; Kapur et al., 2015a; Leontyev et al., 2012; Semple, 2010). However, bone marrow megakaryocyte counts revealed a significant difference among the groups of antibody-treated mice. For example, although both anti-β3 sera and RAMS induced comparable levels of thrombocytopenia in recipient mice, the megakaryocyte count was significantly higher only in the anti- β3 sera or MWReg30 treated groups (Figure 2.2 A). This may suggests that for
some anti-platelet antibodies, the megakaryocyte response in ITP is not necessarily proportional to peripheral platelet counts. The difference in megakaryocyte counts between the anti-β3 sera and RAMS may also be related to the differences in the antigen specificities or the Fc structure of the antibodies in the sera. Moreover, the megakaryocyte counts of the MWReg30 treated mice were the highest among all groups while the platelet counts of these mice were significantly reduced (Figures 2.1 and 2.2). This may suggest a distinctive effect that MWReg30 has both on platelets and megakaryocytes in murine passive ITP. Considering the antigen specificities of the antibodies, it seems possible that the interaction between MWReg30 and its cognate antigen CD41 could stimulate megakaryocyte progenitors to promote the production of megakaryocytes. Support for this, at least, is that the binding of MWReg30 to CD41 significantly increases platelet activation whereas other anti-GPIbIIIa (CD41/CD61) mAbs actually inhibit platelet activation (Burstein et al., 1992; Di Minno et al., 1983). Alternatively, it might also be possible that the difference between MWReg30 and other mAbs are due to an inhibitory effect of anti-GPIb (CD42) mAbs on megakaryocyte differentiation or proliferation. There is supportive evidence from the study by Balduini et al showing that anti-GPIb antibody but not anti-GPIIbIIIa antibody inhibited proplatelet formation from megakaryocytes in vitro (Balduini et al., 2008).

Comparisons of the four different anti-GPIb mAbs including NIT-B, NIT-E, NIT-G, NIT-H1 showed comparable numbers of megakaryocytes in the bone marrow, despite a trend of higher platelet counts in the NIT-E-treated group (Figure 2.1 A), suggesting that the number of megakaryocytes in the bone marrow may not be sensitive to increased platelet destruction and thrombocytopenia. Similarly, others have shown that the number of megakaryocytes and progenitors does not significantly change within seven days after an anti-platelet serum injection (Ebbe et al., 1987; Stenberg and Levin, 1989). However, in these studies there was an increase of the size of megakaryocytes, which needs to be further confirmed in our mouse model (Ebbe et al., 1987; Stenberg and Levin, 1989). Overall, the different megakaryocyte counts between MWReg30 and the other mAbs, and the similarities among anti-GPIb antibodies suggest that the antigen specificity of Abs may determine their effect on megakaryocyte differentiation and proliferation. This may help explain the variability in megakaryocyte counts in ITP patients with counts varying from normal to elevated (Dameshek and Miller, 1946; De La Fuente, 1949;
In our study, IVIg treatment successfully increased platelet counts in all groups except mice receiving the NIT-E mAb (Figure 2.2 B). Of interest, there were no significant changes in the megakaryocyte counts in the IVIg-treated mice, except in the MWReg30-treated group. Our study confirms the effectiveness of IVIg therapy in the passive ITP mouse model (Leontyev et al., 2012; Samuelsson et al., 2001; Semple, 2010), but suggests that IVIg may not affect megakaryocyte counts in the bone marrow.

In summary, anti-platelet antibodies, independent of anti-platelet T cell-mediated destruction, are able to induce thrombocytopenia but most do not affect megakaryocyte counts or morphology within the bone marrow in 24 hours. Our data suggest that while the thrombocytopenia induced by platelet antibodies is generally sensitive to IVIg treatment, in those mice where megakaryocyte number and morphology are affected, IVIg does not necessarily change the effect.
Chapter 3

Allogeneic Platelet Transfusions Prevent Murine T Cell-mediated ITP

This chapter is modified from the following:
3.1 Abstract

Platelet transfusions are lifesaving treatments for many patients with thrombocytopenia, however, their use is generally discouraged in the autoimmune disorder immune thrombocytopenia (ITP). We examined whether allogeneic platelet major histocompatibility complex (MHC) class I transfusions affected anti-platelet CD61-induced ITP. BALB/c CD61 knockout (KO) mice (CD61+/H-2d) were immunized against platelets from either wildtype syngeneic BALB/c (CD61+/H-2d) or allogeneic C57BL/6 (CD61+/H-2b) or C57BL/6 CD61 KO (CD61+/H-2b) mice and their splenocytes were transferred into severe combined immunodeficient (SCID) mice to induce ITP. When non-depleted splenocytes were transferred to induce antibody-mediated ITP, both allogeneic and syngeneic CD61+ platelet immunizations generated immunity that caused thrombocytopenia independently of allo-MHC molecules. In contrast, when B cell-depleted splenocytes were transferred to induce T cell-mediated ITP, transfer of allogeneic MHC-immunized splenocytes completely prevented CD61-induced ITP development. In addition, allogeneic platelet transfusions into SCID mice with established CD61-induced ITP rescued the thrombocytopenia. Compared with thrombocytopenic mice, bone marrow histology in the rescued mice showed normalized megakaryocyte morphology and in vitro CD61-specific T cell cytotoxicity was significantly suppressed. These results indicate that antibody-mediated ITP is resistant to allogeneic platelet transfusions whereas the T cell-mediated form of the disease is susceptible suggesting that transfusion therapy may be beneficial in antibody-negative ITP.
3.2 Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by an isolated thrombocytopenia defined as a peripheral blood platelet count of less than $100 \times 10^9$/L (Provan et al., 2010; Rodeghiero et al., 2009). The pathogenesis of ITP is complex but appears to be due primarily to IgG-mediated peripheral platelet destruction in the spleen and/or bone marrow megakaryocyte inhibition/destruction (Chang et al., 2003; Cines et al., 2009; Harrington et al., 1951; McMillan et al., 2004; Shulman et al., 1965). A second mechanism of immune thrombocytopenia has been suspected, however, as early as the famous 1951 Harrington experiments where they observed that only 16 of the 26 ITP (62%) plasma infusions into healthy volunteers caused thrombocytopenia. Subsequently, the cumulative results of antibody studies in patients with ITP revealed that anti-platelet antibodies can only be identified in approximately 60% of patients with ITP (Brighton et al., 1996; Fabris et al., 2004; Kiefel et al., 1996; McMillan, 2003; Najaoui et al., 2012). In those patients with no identifiable antibodies, Olsson et al elegantly demonstrated that cytotoxic T cell (CTL) cytotoxicity was responsible for the thrombocytopenia and this was subsequently confirmed by other studies in both humans and animals with ITP (Chow et al., 2010; Olsson et al., 2003; Zhang et al., 2006; Zhao et al., 2008). The latter ITP animal model also demonstrated that compared with antibody-mediated ITP, the T cell-mediated form of the disorder was not sensitive to platelet-sparing therapies such as intravenous gammaglobulin (IVIg) (Chow et al., 2010).

Therapy for patients with ITP generally includes steroids and/or IVIg and if those fail, other treatments such as rituximab, thrombopoietin (TPO) receptor agonists and/or splenectomy etc. are available (Provan et al., 2010; Rodeghiero et al., 2009). Of interest, despite the benefit of platelet transfusions for many thrombocytopenic conditions such as the thrombocytopenia secondary to leukemia (Han et al., 1966; Hirsch and Gardner, 1952; Slichter, 1980), the use of allogeneic platelets in patients with ITP has been generally withheld (Carr et al., 1986; Salama et al., 2008; Spahr and Rodgers, 2008; Thachil, 2010). Allogeneic platelet transfusions for chronic ITP are recommended only as emergency treatment and to be used in combination with other treatments such as IVIg (Provan et al., 2010; Rodeghiero et al., 2009). Platelet transfusions in ITP are generally avoided due to concerns regarding the ineffectiveness of the treatment or possible adverse reactions (Thachil, 2010). However,
when one examines the literature for evidence supporting a lack of benefit for platelet transfusions in ITP, very few reports are in fact published. Those that are published are mostly case reports but more than half of them actually show a benefit from platelet transfusions (satisfactory corrected count increment) in patients with ITP (Carr et al., 1986; Salama et al., 2008; Spahr and Rodgers, 2008; Thachil, 2010).

Our murine model of active CD61-specific ITP demonstrate both antibody- and T cell-mediated thrombocytopenia and because the T cell-mediated form of thrombocytopenia was resistant to IVIg (Chow et al., 2010), we examined whether allogeneic platelet transfusions may have a differential effect on the two immunopathologic forms of ITP. The results show that although antibody-mediated ITP is not affected by allogeneic platelet transfusions, the T cell-mediated form of the disorder was alleviated by the transfusions. This suggests that platelet transfusions may be beneficial in individuals suffering from anti-platelet antibody negative ITP.
3.3 Materials and Methods

3.3.1 Mice

Female BALB/c (CD61\(^{+}\)/H-2\(^{b}\)), C57BL/6 (CD61\(^{+}\)/H-2\(^{b}\)) and B6.129S2-Itgb3\(^{tm1Hyn}\)/J CD61 knockout (KO) (CD61\(^{-}\)/H-2\(^{b}\)) mice, 8-12 weeks of age, were used as platelet donors and were obtained from The Jackson Laboratories. BALB/c CD61 KO mice (CD61\(^{-}\)/H-2\(^{d}\)) were bred in the laboratory of Dr. Heyu Ni and used as platelet recipients and the source of immune splenocytes. Female CB.17 severe combined deficient SCID (H-2\(^{d}\), CB17/Icr-\(Prkdc^{scid}/IcrIcoCrl\)) mice (8-12 weeks of age) were used as spleen-cell transfer recipients for induction of ITP and were obtained from Charles River. All mice were housed in the Li Ka Shing Knowledge Institute’s Research Vivarium and all animal studies were approved by the St. Michael’s Hospital Animal Care Committee.

3.3.2 Platelet preparation and immunization of CD61 KO mice

Leukoreduced platelets from the indicated donor mice (Table 3.1) were prepared as previously described (Chow et al., 2010). Briefly, blood was collected from the indicated donor mice, diluted with 1 × phosphate buffered saline (PBS) containing 10% citrate-phosphate-dextrose with adenine (PBS/CPDA buffer) and centrifuged at 120 × g. Platelet-rich plasma was then collected and washed at 450×g. The washed platelets were resuspended in PBS, adjusted to 1×10\(^{9}\) cells/mL and 100 uL was transfused into BALB/c CD61 KO (CD61\(^{-}\)/H-2\(^{d}\)) mice weekly for 4 weeks.

3.3.3 Serum anti-CD61 and anti-MHC class I antibody production

Serum IgG anti-CD61-specific or anti-platelet MHC class I-specific antibodies were detected by flow cytometry. Briefly, 1×10\(^{6}\) leukoreduced platelets from the indicated donor mice were incubated with titrations of serum from the indicated immune BALB/c CD61 KO mice for 30 minutes at room temperature. The platelet-serum mixtures were washed once with PBS/CPDA buffer and then labeled with a FITC conjugated goat anti-mouse IgG antibody (Fc specific; Caltag Laboratories, Mississauga, ON) in the dark at room temperature for 30 minutes.
Table 3.1 Summary of platelet populations, immune splenocytes used and expected immune responses induced.

<table>
<thead>
<tr>
<th>Platelet Source</th>
<th>Platelet/MHC Phenotype</th>
<th>Mice Immunized (Splenocyte Source)</th>
<th>Expected Immune Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice</td>
<td>CD61^+ /H-2^b</td>
<td>BALB/c CD61 KO mice</td>
<td>IgG anti-CD61 antibodies. IgG anti-MHC antibodies.</td>
</tr>
<tr>
<td>C57BL/6 CD61 KO mice</td>
<td>CD61^- /H-2^b</td>
<td>BALB/c CD61 KO mice</td>
<td>IgG anti-MHC antibodies.</td>
</tr>
</tbody>
</table>
The mixture was washed with PBS/CPDA buffer and acquired and analyzed on a by flow cytometer (BD FACSsort, Becton Dickinson, Mississauga, ON).

### 3.3.4 Preparation of splenocytes and cell depletion

Immune CD61 KO mice (CD61−/H-2d) were sacrificed and their spleens removed, homogenized in RPMI-1640 medium and washed twice by centrifugation at 400 × g for 15 minutes. Splenocyte suspensions were further treated with ammonium-chloride-potassium red blood cell (RBC) lysing solution and washed with 1 × PBS to remove lysed RBC. Cell depletion studies were carried out using the Stem Cell Technologies EasySep mouse CD19 positive selection kit (Catalog No. 18754), according to the manufacturers instructions. Briefly, splenocytes were combined with the labeling reagent and PE selection cocktail and magnetic particles were added and placed within a magnetic field to isolate CD19+ cells. The unbound CD19− cells were resuspended in RPMI-1640 medium at the concentration of 1.5×10⁵/mL and CD19 depletion efficiencies were determined by flow cytometry. CD19+ cells were less than 1% of all splenocytes after depletion.

### 3.3.5 ITP induction

SCID mice were pre-screened for the presence of serum IgG by an enzyme-linked immunoabsorbent assay (ELISA) and any mouse with a serum IgG concentration greater than 20μg/mL was deemed “leaky” and excluded from study. ITP was induced as previously described (Chow et al., 2010). Briefly, on day -1, CB.17 SCID mice were bled via the saphenous vein and pre-treatment platelet counts were measured using a Beckman Coulter Counter-LH750 hematology analyzer. NK cells were depleted in the mice by an ip infusion of 50 uL of a rabbit anti-asialo GM1 antibody (Wako Pure Chemical Industries, Ltd). On day 0, the mice were sub-lethally gamma irradiated (200 cGy) and then received 100 uL (1.5 ×10⁴ cells total) of the non-depleted or CD19-depleted immune splenocytes (ip) from the indicated immune CD61 KO mice. Peripheral blood of SCID mouse recipients was collected weekly from the saphenous vein and platelet counts were measured. The protocol for ITP induction is shown in Figure 3.1 and expected immune responses are shown in Table 3.1.
1. BALB/c CD61 KO mice were transfused weekly with one of the 3 indicated platelet populations and immunity was confirmed at day 28 by the generation of anti-CD61 and/or anti-MHC serum antibodies (IgG+). 2. The immune KO mice were sacrificed and their splenocytes were either transferred ip to CB.17 SCID mice and weekly platelet counts and phenotype were monitored or they were assessed for in vitro cytotoxicity. 3. At day 28 post-splenocyte transfer, the SCID mice were sacrificed and effector responses (in vitro cytotoxicity) and bone marrow (BM) histology were performed.
3.3.6 Bone marrow histology and megakaryocyte counts

Engrafted SCID mice were sacrificed on day 28 and their femurs were removed, dissected of muscle tissue and the epiphyses were cut off and the bones shafts were placed in fixative (B+ fixative; BBC Biochemicals) for 12 hours. The bones were further decalcified with 10% nitric acid for 2 hours and then processed and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). All pictures were taken by using CellSens Standard software under a 40× objective lens (Olympus BX50), with the numerical aperture of objective lens 0.75. The megakaryocyte count of each mouse was calculated as the mean value of 10 counts from 10 random fields (450μm×320μm/field) throughout each slide.

3.3.7 Cytotoxicity assay

Anti-CD61 cytotoxic activity of splenocytes from the transfused CD61 KO mice and engrafted SCID mice was measured using a commercial kit (7-AAD/CFSE Cell-Mediated Cytotoxicity Assay Kit, Cayman Chemical company, item no. 600120) and the murine macrophage cell line PU5-1.8 (H-2^d) was used as a target cell (ATCC, TIB-61) as this cell line expresses both syngeneic MHC class I and CD61 antigens (determined by flow cytometry, not shown). Briefly, target cells were stained with 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) for 15 min at room temperature washed once with RPMI-1640 medium and 10^4 cells were incubated in 96-well V-bottom plates with the indicated splenocytes (effectors) for 4 h at 37 ºC at different Effector:Target ratios (1.25:1, 2.5:1, 5:1 and 10:1). The plates were then centrifuged and the cells resuspended in 7-AAD staining solution to label dead cells. The samples were analyzed by flow cytometry (MACSQuant Analyzer, MiltenyiBiotec) and cytotoxic activity was evaluated by analyzing the percentage of dead cells within the CFSE-labeled target cell population. To address the effects of platelets on splenic cytotoxicity, 10^5 splenocytes were first incubated with either BALB/c WT (CD61+/H-2^d) or C57BL/6 WT (CD61+/H-2^d) platelets at a 1:15 splenocyte: platelet ratio in V-bottom plates for 4h at 37 ºC, washed twice with RPMI-1640 medium and then used as effectors in the cytotoxicity assay with labeled PU5-1.8 target cells.
3.3.8 Statistical analysis

Differences between means were analyzed with Student’s $t$ test and $P$ values of < .05 were considered significant.
Figure 3.2 Generation of anti-platelet IgG antibodies in CD61 KO mice.

Representative flow cytometric histograms of anti-CD61 and/or anti-MHC class I serum reactivity from immunized BALB/c CD61 KO mice. BALB/c CD61 KO mice were weekly transfused (×4) with platelets from either (a) BALB/c (CD61+/ H-2d) mice, (b) C57BL/6 (CD61+/ H-2b) mice or (c) C57BL/6 CD61 KO (CD61−/ H-2b) mice. Serum was prepared and incubated (1:400 dilution shown) with either BALB/c platelets (top panels), C57BL/6 platelets (middle panels) or C57BL/6 CD61 KO platelets (lower panels) and then incubated with a FITC-conjugated goat anti-mouse IgG secondary antibody and fluorescence was analyzed by flow cytometry. The thin line in each histogram is the pre-treatment serum reactivity and the thick lines are serum reactivity after 4 platelet transfusions. Splenocytes from the immune mice were then used in the indicated experiments.
3.4 Results

3.4.1 Platelet MHC class I and CD61 antigens generate high titred IgG antibody responses

When BALB/c CD61 KO mice were immunized with either (a) BALB/c (CD61\(^{+}\)/H\(-2^{d}\)), (b) C57BL/6 (CD61\(^{+}\)/H\(-2^{b}\)), or (c) C57BL/6 CD61 KO (CD61\(^{-}\)/H\(-2^{b}\)) platelets, significant levels of anti-platelet IgG antibodies against the corresponding CD61 and/or allogeneic MHC class I antigens were detected in the sera after the fourth platelet transfusion, i.e. (a) anti-CD61 antibodies, (b) anti-CD61 and MHC antibodies and (c) anti-MHC antibodies respectively (Figure 3.2).

3.4.2 Platelet allogeneic MHC class I antigens do not affect anti-CD61 antibody-mediated ITP

The effect of allogeneic MHC class I molecules on CD61-specific IgG-mediated ITP was determined by transferring non-depleted splenocytes from CD61 KO mice immunized against the three platelet populations into SCID mice and then monitoring platelet counts weekly. As described previously (Chow et al., 2010), irradiation-induced thrombocytopenia occurred at day 7 in all mice (Figure 3.3). Compared with SCID mice transferred with naïve mouse splenocytes, non-depleted splenocytes from CD61 KO mice immunized against either CD61\(^{+}\)/H\(-2^{d}\) or CD61\(^{+}\)/H\(-2^{b}\) platelets induced significant thrombocytopenia throughout the 28 day protocol (day 21, 28, \(P<.05\)) (Figure 3.3a).

3.4.3 Platelet allogeneic MHC class I antigens prevent anti-CD61 T cell-mediated ITP

To determine whether allogeneic MHC class I molecules have an effect on CD61-specific T cell-mediated ITP, splenocytes from the CD61 KO mice immunized with the three platelet populations were first depleted of CD19\(^{+}\) B cells and then transferred into SCID mice. SCID mice transferred with CD19-depleted immune splenocytes against CD61\(^{+}\)/H\(-2^{d}\) platelets developed thrombocytopenia throughout the 28 day protocol (Figure 3.3b). However, SCID mice transferred with CD19-depleted
Figure 3.3 Allogeneic platelet MHC I antigens inhibit T cell-mediated ITP.

Platelet counts in SCID mice transferred with either (a) non-depleted or (b) CD19-depleted splenocytes from either naïve BALB/c mice (●, N=18) or from BALB/c CD61 KO mice immunized against platelets from BALB/c (CD61+/ H-2<sup>d</sup>) mice (○, N=11), C57BL/6 (CD61+/ H-2<sup>b</sup>) mice (△, N=18) or C57BL/6 (CD61-/ H-2<sup>b</sup>) CD61 KO mice (□, N=8). Results are presented as weekly platelet counts (×10<sup>9</sup>/L) after splenocyte transfer. The dotted horizontal line represents the mouse platelet cut-off counts for thrombocytopenia. In a) *; p<0.05 for □ vs. △ or ○) and in b) *; p<0.05 for △ vs. ○.
immune splenocytes against CD61+/H-2\textsuperscript{b} platelets were rescued from the T cell-mediated thrombocytopenia at days 21 and 28 ($P<.01$) (Figure 3.3b).

### 3.4.4 Allogeneic platelet transfusions prevented T cell-mediated ITP in transferred SCID mice

To ensure that allogeneic platelet transfusions would prevent an established T cell-mediated ITP, SCID mice were first transferred with ITP-inducing CD19-depleted CD61 KO immune splenocytes against BALB/c (CD61$^+$/H-2\textsuperscript{d}) platelets, and then transfused weekly with either $10^8$ BALB/c syngeneic or C57BL/6 allogeneic platelets. Compared with control non-transfused mice, platelet counts in ITP SCID mice transfused with allogeneic C57BL/6 platelets were significantly elevated (day 28, $P < .05$) (Figure 3.4).

### 3.4.5 Allogeneic platelet MHC class I molecules reverse abnormal bone marrow morphology and numbers in anti-CD61 T cell-mediated ITP

Compared with SCID mice transferred with naïve splenocytes (Figure 3.5a), CD19-depleted splenocytes transferred from CD61 KO mice immunized against BALB/c (CD61$^+$/H-2\textsuperscript{d}) platelets caused significant abnormalities in the SCID mouse bone marrow megakaryocytes (Figure 3.5b). Megakaryocytes in those mice had increased size, pyknotic nuclei, and irregular membranes (Figure 3.5b). In contrast, however, megakaryocytes in the SCID mice receiving CD19-depleted CD61 KO immune splenocytes against either C57BL/6 (CD61$^+/H-2^b$) or C57BL/6 CD61 KO (CD61$^+/H-2^b$) platelets showed no difference in bone marrow histology when compared with the control mice (Figure 3.5c and d). Cumulatively, the numbers of megakaryocytes were significantly increased in mice exhibiting antibody-mediated ITP, whether induced by splenocytes from CD61 KO mice immune against CD61$^+/H-2^d$ or CD61$^+/H-2^b$ platelet populations (Figure 3.6). However, in mice with T cell-mediated thrombocytopenia, i.e. the SCID mice that received CD19-depleted immune splenocytes, the presence of MHC antigens on platelets significantly reduced the number of megakaryocytes to normal levels (Figure 3.6).
Figure 3.4 Allogeneic platelet transfusions inhibit T cell-mediated ITP.

Platelet counts in SCID mice transferred with CD19-depleted CD61 KO immune splenocytes against BALB/c (CD61+/H-2^d) platelets and then transfused weekly with either nothing (○, N=5), syngeneic BALB/c platelets (▼, N=5) or allogeneic C57BL/6 platelets (□, N=5). Results are presented as the weekly platelet counts (×10^9/L) after splenocyte transfer (★; p<0.05 for □ vs. ○).
3.4.6 Allogeneic platelet MHC class I molecules inhibit CD61-specific T cell cytotoxicity in vitro

To further examine the relationship between platelet counts and the anti-CD61 T cell response, the cytotoxic activity of splenocytes from immunized CD61 KO mice and the transferred SCID mice were examined using an in vitro cytotoxicity assay against syngeneic CD61+ target cells (PU5-1.8) (Figure 3.7a). Splenocytes from CD61 KO mice immunized with BALB/c (CD61+/H-2d) platelets showed significantly elevated anti-CD61 cytotoxicity at an Effector: Target ratio of 10:1 as compared with control naïve splenocytes (P<.01) (Figure 3.7b). This cytotoxicity was also observed in the spleens of SCID mice receiving those immune CD61 KO splenocytes (Figure 3.7c). In contrast, the presence of allogeneic platelet MHC molecules (CD61+/H-2b) significantly inhibited the anti-CD61 T cell mediated cytotoxicity to levels similar to control naïve splenocytes in both CD61KO mice and their SCID recipients at an Effector: Target ratio of 10:1 (CD61+/H-2b VS CD61+/H-2d, P<.05) (Figure 3.7b and c). To directly address the modulatory effect of allogeneic platelets on the anti-CD61 T cell mediated cytotoxicity, BALB/c CD61 KO mice splenocytes (CD61+/H-2d) immunized against syngeneic WT platelets (CD61+/H-2d) were first incubated with either syngeneic (CD61+/H-2d) or allogeneic (CD61+/H-2b) platelets and then examined for their cytotoxic activity against PU5-1.8 cells. Compared with splenocytes incubated with syngeneic platelets, those with allogeneic platelets showed significantly decreased cytotoxic activity (P<.05) (Figure 3.7d).
Figure 3.5 Allogeneic platelet MHC I antigens rescue megakaryocyte abnormalities in T cell-mediated ITP.

Representative H&E stained bone marrows of SCID mice transferred with CD19-depleted splenocytes from (a) a control BALB/c naïve mouse or BALB/c CD61 KO mice immunized against (b) BALB/c (CD61+/ H-2d) platelets, (c) C57BL/6 (CD61+/ H-2b) platelets or (d) C57BL/6 CD61 KO (CD61-/ H-2b) platelets. The bars in each panel represent 50 μm. Pictures were taken at 40× magnification with a 0.75 numerical aperture of the objective lens for all panels. The yellow arrows point to megakaryocytes.
Figure 3.6 Allogeneic platelet MHC I antigens normalize the number of megakaryocytes in T cell-mediated ITP.

Cumulative results of megakaryocyte enumeration in the bone marrow of control naïve SCID mice (hatched column) or SCID mice transferred with either non-depleted (ND) splenocytes (antibody-mediated ITP) from the indicated platelet donors (white columns) or SCID mice transferred with CD19-depleted splenocytes (T cell-mediated ITP) from the indicated platelet donors (black columns). At least 10 fields in each bone marrow were counted and the results are presented as the mean megakaryocyte number per field (***; P<.001).
Figure 3.7 Effect of allogeneic platelet MHC I antigens on in vitro cytotoxicity in antibody- and T cell-mediated ITP.

a. Naive 15.08%  
CD61+/H-2d 52.70%  
CD61+/H-2b 18.20%  

CFSE

b. c.
(a) Representative flow cytometric dot plot analysis of cytotoxic killing against CD61+ H-2^d PU5-1.8 target cells by non-depleted splenocytes from either a naïve BALB/c mouse (left panel), or from CD61 KO mice immunized either against BALB/c (CD61+/ H-2^d) platelets (middle panel) or against C57BL/6 (CD61+/ H-2^b) platelets (right panel). Splenocytes were incubated with CFSE-labeled CD61+ H-2^d PU5-1.8 cells for 4 hours in vitro and then stained with the vital dye 7-AAD and acquired on a flow cytometer. Results are shown at an Effector:Target ratio of 10:1. (b) Cumulative cytotoxicity of splenocytes from naïve mice BALB/c mice (●, N=19) or CD61 KO mice transfused weekly with 10^8 platelets from either BALB/c (CD61+ H-2^d, ○, N=10) or C57BL/6 (CD61+/ H-2^b, △, N=10) mice. (c) Cumulative cytotoxicity of splenocytes from naïve mice BALB/c mice (●, N=19) or SCID mice transferred with the corresponding CD61 KO immune splenocyte populations as in (b) at 4 weeks post transfer. Results in b and c are expressed as Percent Lysis at the indicated Effector:Target ratios (★★; p<0.01 for ○ vs. △).

(d) Splenocytes from BALB/c CD61 KO mice immunized against platelets from BALB/c (CD61+/H-2^d) mice were incubated with either syngeneic BALB/c WT platelets (CD61+/H-2^b) or allogeneic C57 BL/6 WT platelets (CD61+/H-2^b) for 4h in vitro and then examined for their cytotoxicity against PU5-1.8 target cells. Results in d are expressed as Percent Lysis at a 5:1 Effector:Target Ratio (N=3)★; p<0.05).
3.5 Discussion

Platelet transfusions are generally not indicated as a treatment for ITP although the data supporting this contention are rather scarce in the literature. Since ITP is caused by at least two distinct immune effector processes e.g. antibody- and/or T cell-mediated thrombocytopenia, it is possible that these immunopathologies may have differential responses to therapy. This is supported by an animal model of ITP that demonstrated while antibody-mediated thrombocytopenia was sensitive to IVIg treatment, T cell-mediated thrombocytopenia was not (Chow et al., 2010). In the current murine ITP study we have further elucidated the effect of allogeneic platelet transfusions on antibody- and T cell-mediated ITP. Immunization by platelet transfusions containing both allogeneic MHC class I molecules and CD61 molecules (CD61+/H-2b) rendered the spleens of CD61 KO mice (CD61−/H-2d) incapable of mediating anti-CD61-specific T cell-mediated ITP in SCID mice. This was also true if SCID mice that had established T cell-mediated ITP and were transfused with allogeneic platelets. These responses were correlated with a normalization of bone marrow megakaryocytes and inhibition of in vitro T cell-mediated cytotoxicity. The results suggest that platelet transfusions may be a beneficial therapy in those anti-platelet antibody negative individuals that exhibit T cell-mediated ITP.

In 2003, Olsson et al showed that in patients with active ITP who did not have any identifiable anti-platelet antibodies, there was a significantly increased cytotoxic activity by peripheral blood T cells against self platelet antigens (Olsson et al., 2003). This activity was reduced in those patients whose platelet counts increased either due to therapy or spontaneously and this was also reproduced in a larger clinical trial and in animal studies (Chow et al., 2010; Zhang et al., 2006; Zhao et al., 2008). In our current murine study, analogous results were observed in that CD19-depleted CD61 KO immune splenocytes against CD61+/H-2d platelets induced significant thrombocytopenia when transferred into SCID mice (Figure 3.3). This correlated to enhanced T cell cytotoxicity against CD61+ syngeneic target cells in vitro (Figure 3.7 b and c). This indicates that murine platelet CD61 antigens can effectively trigger CTL responses similar to patients with ITP (Chow et al., 2010; Olsson et al., 2003; Zhang et al., 2006; Zhao et al., 2008).
SCID mice transferred with CD19-depleted splenocytes from CD61 KO mice (CD61+/H-2^d) that were immunized with allogeneic CD61^+/H-2^b platelets did not develop thrombocytopenia (Figure 3.3). This correlated with a loss of in vitro cytotoxicity against CD61^+ target cells (Figure 3.7). Furthermore, in SCID mice transferred with CD19-depleted splenocytes from CD61 immune KO mice and then transfused weekly with allogeneic platelets (CD61^+/H-2^b), their platelet counts were significantly rescued compared with control ITP mice (Figure 3.4). The mechanism of how cytotoxic T cell inhibition and a rescue of platelet counts occur is unknown but some possibilities exist. For example, CTL function by inducing target cell cytolysis via apoptosis that is initiated by the interaction between their T cell receptor (TCR) complex and MHC class I/peptide complexes on target cells (Bousso and Robey, 2003; Kaech and Ahmed, 2001). Platelet MHC class I molecules are primarily adsorbed from plasma and consist of truncated heavy chains that are unstable, indicated by the finding that more than 80% can be eluted from the platelet surface by chloroquine diphosphate without affecting platelet membrane integrity (Blumberg et al., 1984; Ghio et al., 1999; Kao, 1987, 1988; Kao et al., 1986; Neumuller et al., 1993). Moreover, platelet MHC class I molecules can be partially stabilized by addition of exogenous β2-microglobulin in vitro, indicating a reduction of the β2-microglobulin molecule on the platelet surface (Gouttefangeas et al., 2000). Because of these features, allogeneic platelet MHC class I molecules may thus evoke a direct faulty interaction with the CTL TCR thereby anergizing the effector cell. Support for this comes from studies that show that allogeneic platelet MHC class I molecules cannot activate CTLs on their own (Gouttefangeas et al., 2000) and our previous work demonstrating that allogeneic platelet transfusions can significantly enhance donor-specific skin graft survival, an immune process that is almost exclusively elicited by CTL (Aslam et al., 2008). Figure 3.7 d shows that the in vitro CTL cytotoxicity could be prevented by allogeneic platelets whereas syngeneic platelets had no effect supporting the concept that T cell-mediated ITP may be inhibited by the direct interaction of T cells with allogeneic platelets. On the other hand, the denatured platelet MHC class I molecules may be processed within antigen presenting cells (APCs) in a manner that does not allow for proper cross presentation of platelet allogeneic MHC peptides to the CTL. We are currently studying this.
The ITP in mice, whether induced by antibodies or T cells, showed abnormal bone marrow histology; megakaryocytes were increased in number and they showed evidence of apoptosis e.g. pyknotic nuclei (Figure 3.5). These data support the findings in human studies that patients with ITP have elevated numbers of megakaryocytes that exhibit apoptosis-like qualities (Houwerzijl et al., 2004; Li et al., 2007; Yang et al., 2010) and this concurs with in vitro assays that show that anti-platelet antibodies significantly inhibit megakaryocyte growth (Chang et al., 2003; McMillan et al., 2004). The increased number of megakaryocyte in this model may be a compensation response to the thrombocytopenia and immune attack causing apoptosis of megakaryocytes. Perhaps more intriguing, however, is the observation that when mice were rescued of T cell-mediated ITP by allogeneic platelet transfusions, their bone marrow megakaryocyte histology was similar to control mice (Figure 3.5). This suggests that inhibiting the anti-platelet T cell cytotoxic response by allogeneic transfusions may relieve the megakaryocytes from T cell attack and enable them to maintain platelet production.

Future applications of these murine findings in humans will require the prerequisite of screening for anti-platelet antibodies which may pose an obstacle e.g. requirement of quick turnaround times and potential assay sensitivity issues. Nonetheless, the results presented here suggest that in at least a proportion of individuals that are antibody negative, allogeneic platelet transfusions may have a beneficial role in alleviating ITP.

In summary, our study shows that anti-CD61 specific T cell-mediated immune responses can induce thrombocytopenia in SCID mice recipients, mimicking the situation observed in antibody negative ITP patients. The cell-mediated ITP could be abolished by transfusions of allogeneic platelet MHC I antigens indicating a potential immunomodulatory benefit of allogeneic platelet transfusions in ameliorating T cell-mediated ITP.
Chapter 4

CD20\(^+\) B Cell Depletion Therapy Suppresses Murine CD8\(^+\) T Cell-mediated Immune Thrombocytopenia (ITP)

This chapter is modified from the following:

4.1 Abstract

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder with a complex pathogenesis which includes both antibody-mediated and T cell-mediated effector mechanisms. Rituximab (an anti-human CD20 monoclonal antibody) is one of the treatments for ITP and is known to deplete B cells but may also work by affecting the T cell compartments. Here we investigated the outcome of B cell depletion (Bdep) therapy on CD8⁺ T cell-mediated ITP using a murine model. CD61 knockout (KO) mice were immunized with CD61⁺ platelets and T cell-mediated ITP was initiated by transfer of their splenocytes into severe combined immunodeficient (SCID) mice. The CD61 KO mice were administrated an anti-mouse CD20 monoclonal antibody either before or after CD61⁺ platelet immunization. This resulted in efficient Bdep in vivo, accompanied by significant increases in splenic and lymph node CD4⁺ and CD8⁺ T cells and proportional increases of FOXP3⁺ in CD4⁺ and CD8⁺ T cells. Moreover, Bdep therapy resulted in significantly decreased splenic CD8⁺ T cell proliferation in vitro that could be rescued by interleukin-2 (IL-2). This correlated with normalization of in vivo platelet counts in the transferred SCID mice suggesting that anti-CD20 therapy significantly reduces the ability of CD8⁺ T cells to activate and mediate ITP.
4.2 Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by an isolated thrombocytopenia (<100 ×10^9/L) (McKenzie et al., 2013). The pathogenesis of chronic ITP is incompletely understood and thought to be heterogeneous (McKenzie et al., 2013). For example, approximately two thirds of patients with ITP have detectable anti-platelet antibodies that destroy platelets primarily by Fc-mediated phagocytosis within the spleen (Brighton et al., 1996; Harrington et al., 1951). On the other hand, patients with no detectable antibodies harbor platelet- and megakaryocyte-specific T cells that can mediate ITP (Li et al., 2007; Olsson et al., 2003). These effector responses are closely correlated with dysfunctional CD4⁺ T regulatory cells (Tregs) suggesting T cells are critical for ITP development (Liu et al., 2007; Sakakura et al., 2007; Stasi et al., 2008).

B cell depletion (Bdep) therapy using rituximab has been shown to be a successful second-line treatment for patients with ITP (Stasi et al., 2001). Although B cells function to primarily elicit humoral immunity, they also have other roles such as antigen presentation, co-stimulation and T cell activation/cytokine production (Lund, 2008; Lund and Randall, 2010). Moreover, patients with ITP, whether anti-platelet antibody positive or not, can respond to rituximab and in those antibody-positive patients, their autoantibody titers do not necessarily change (Cooper et al., 2012). This suggests that anti-CD20 can significantly affect T cell compartments and this was originally confirmed by Stasi et al showing that rituximab normalizes the observed CD4⁺ T cell abnormalities in ITP (Stasi et al., 2008; Stasi et al., 2007). To better understand how anti-CD20 therapy affects CD8⁺ T cells, we investigated its effect on T cell-mediated ITP in an established murine model of ITP (Chow et al., 2010). The data suggest that anti-CD20 Bdep significantly inhibits IL-2 dependent CD8⁺ T cell proliferation which blocks their ability to mediate ITP.
4.3 Materials and Methods

4.3.1 Mice

BALB/c mice (H-2^d, CD61^+, Charles River Laboratories, Montreal, QC, Canada) were used as platelet donors and BALB/c CD61 (GPIIIa) knockout (KO) mice were supplied by Dr. Heyu Ni and used as a source of immune splenocytes. CB.17 severe combined immune deficient (SCID) mice (H-2^d, CB17/Lcr-Prkdc^scid/LcrIcoCrl, Charles River Laboratories) were used as splenocyte transfer recipients for induction of ITP. All mice were 8-12 weeks of age and all studies were approved by the St. Michael’s Hospital Animal Care Committee.

4.3.2 Reagents

The reagents used in our current study include: anti-Mouse CD3 antibody Purified, eBioscience, Cat No. 15-5773-80A; anti-Mouse CD28 Purified, eBioscience, Cat No. 16-0281-85; Mouse IL-2 recombinant protein, eBioscience, Cat No. 14-8021-64; Goat anti-mouse IgG, FITC, Fc specific, Caltag Laboratories, Cat No. M35001; anti-Mouse CD19 Alexa fluor 647, Biolegend, Cat No. 115522; anti-Mouse CD45R/B220 PE, PharMingen, Cat No. 553089; anti-Mouse CD3 PerCP/Cy5.5, Biolegend, Cat No. 100218; anti-Mouse CD4 eFluor450, eBioscience, Cat No. 48-0041-82; anti-Mouse CD4 Antibody Brilliant Violet 510, Biolegend, Cat No. 100559; anti-Mouse CD8 FITC, eBioscience, Cat No. 11-0081-85; anti-Mouse CD8a Antibody Alexa Fluor 647, Biolegend, Cat No. 100724; anti-Mouse/Rat FOXP3 PE-Cy5, eBioscience, Cat No. 15-5773-80A; anti-Human/mouse Granzyme B Antibody Pacific Blue, Biolegend, Cat No. 515407; anti-Mouse CD44 antibody FITC, ThermoFisher Scientific, Cat No. MA5-17879; Mouse CD19 positive selection kit, Stem Cell Technologies, Cat No. 18754; Cell Stimulation Cocktail (plus protein transport inhibitors) (500×), eBioscience, Cat. No. 00-4975-03.

4.3.3 Platelet preparation and immunization of CD61 KO mice

Leukoreduced platelets were prepared from platelet rich plasma as previously described (Chow et al., 2010). Briefly, blood was collected into phosphate-buffered saline (PBS)
containing citrate-phosphate-dextrose-adenine. The blood was centrifuged at 120 × g for 15 minutes, and the platelet rich plasma (PRP) was collected. The PRP was washed and then resuspended in PBS at 1×10⁹ platelets/mL. For the immunization of CD61 KO mice, recipient mice were bled 24 h prior to the first transfusion and then injected weekly for 4 weeks with 100 μL (1×10⁸) of fresh platelets via the tail vein.

4.3.4 Cell preparation and staining

Spleen, thymus and mesenteric lymph nodes were harvested from sacrificed mice and homogenized in RPMI-1640 medium and washed by centrifugation at 400 × g for 15 minutes. Cells were then ammonium-chloride-potassium (ACK) treated to remove red blood cells, enumerated, and adjusted to a concentration of 1×10⁷ cells/mL for antibody staining (Aslam et al., 2012; Guo et al., 2014). For direct cell surface staining, each indicated antibody was used at 0.5-1μL/100 μL, incubated for 30 min at room temperature, washed and resuspended in staining buffer. For FOXP3 staining, as described previously (Aslam et al., 2012), lymphocytes from thymus, spleen or mesenteric lymph nodes were first stained with anti-mouse CD4 eFluor450 and anti-mouse CD8 FITC antibodies for 45 min at 4°C, washed, and then fixed and permeabilized using the FOXP3 staining buffer. The cell preparations were then subsequently stained with anti-mouse FOXP3 PE-Cy5 antibody or isotype IgG control. For Granzyme B staining, purified CD8⁺ T cells from the proliferation cultures (see below) were collected and stained for anti-mouse CD3 PerCP/Cy5.5 and anti-mouse CD8 APC antibodies, fixed and permeabilized, and then stained with anti-mouse Granzyme B Pacific Blue antibody or isotype IgG control. Cells were analyzed using a BD LSRFortessa X-20 flow cytometer (BD Biosciences, USA) or a MACSQuant Analyzer (Miltenyi Biotec).

4.3.5 Cell depletion and isolation in vitro

For in vitro CD19⁺ depletion and CD8⁺ isolation, viable nucleated cells from homogenized spleens were purified based on density using Lympholyte-M cell separation media following the manufacture instructions (Cedarlane, CL5030).
Figure 4.1 Overview of the experimental design.

Schematic overview of the entire experimental design depicted in a timeline, based on the murine model of ITP, including timing of anti-CD20 monoclonal antibody injections, platelet transfusions, organ harvestings, in vitro analysis and platelet count measurements in the various groups.
The CD19+ depletion for adoptive transfer into SCID mice was carried out using the Stem Cell Technologies EasySep kits according to the manufactures instructions (mouse CD19 positive selection kit, no. 18754). Briefly, splenocytes were combined with a CD19 labeling reagent and magnetic particles, and then placed within a magnetic field to remove unbound cell populations. The unbound CD19− cells were collected and resuspended in RPMI-1640 Medium at the concentration of 1.5×10^5/mL for adoptive transfer into SCID mice.

For the purification of the CD8+ T cells for proliferation assays in vitro, splenocytes were labeled with anti-CD3, anti-CD8 antibodies and a viability marker 7-AAD, and sorted on a BD FACS Aria III SORP cell sorter. CD3+CD8+7AAD− T cells were collected.

Depletion and isolation purity was examined by flow cytometry. After magnetic depletion, CD19+ cells were less than 1% of all splenocytes. CD8+T cell sorting purities were more than 95%.

4.3.6 B cell depletion therapy

To deplete B cells in vivo, a monoclonal mouse anti-mouse CD20 IgG2a antibody (αCD20) (Biogen Idec Inc) were administered to the indicated CD61 KO mice at indicated time points intravenously (i.v.), at a dose of 250ug/100uL per mouse (Figure 4.1) (Xiu et al., 2008). The efficiency of B cell depletion after the anti-CD20 antibody injection was evaluated by the examination of residual CD19+CD220+ B cell percentage in the blood after 24h, 48h, and then weekly by flow cytometry, as well as in the spleen and lymph nodes when the mice were sacrificed on day 21.

4.3.7 Serum anti-CD61 antibody production

To determine anti-CD61 antibody production in the immunized CD61 KO mice with or without B cell depletion therapy, sera was collected from weekly and detected for the presence of anti-CD61 IgG antibodies. Briefly, platelets from syngeneic WT mice were used as a positive anti-CD61 binding control and platelets from naïve CD61 KO mice were used as the negative control. The platelets (1×10^6) were incubated with titrations (from 1:100 to 1:12800) of serum
for 30 minutes in a total volume of 200µL at room temperature. The platelet-serum mixtures were washed once with PBS and then labeled with a FITC conjugated goat anti-mouse IgG (FITC-GAM, Fc specific; Caltag Laboratories, Mississauga, ON) in the dark at room temperature for 30 minutes. The mixture was washed with PBS and then analyzed by flow cytometry (BD FACSort flow cytometer, Becton Dickinson).

4.3.8 CD8⁺ T cell proliferation assay in vitro

CD8⁺ T cells were purified as mentioned earlier using a BD LSRFortessa X-20 flow cytometer. Purified CD8⁺ T cells were washed with PBS twice and resuspended at a concentration of 1 ×10⁶/mL and Violet Proliferation Dye 450 (V450) (BD Biosciences, Cat No.562158) was added at a final concentration of 1µM. The tubes were then incubated at 37°C for 15 min following the manufacturer’s instructions. The labeled CD8⁺ T cells were washed twice with RPMI-1640 medium and resuspended finally in complete RPMI-1640 medium (supplemented with 10% fetal bovine serum (FBS), 500 U penicillin/0.5 mg streptomycin and 100 µM 2-mercaptoethanol). A 96-well flat-bottom plate was precoated with 1µg/mL purified anti-mouse CD3 antibody at 4°C overnight and washed with cold PBS before use. CD8⁺ T cells were added to the 96-well plate at 10⁵ cells/well in triplicate. In the indicated groups, purified anti-mouse CD28 antibody was added at a final concentration of 2µg/mL, and IL-2 was added at 0.05µg/mL. The cells were incubated for 72h at 37°C and then analyzed by flow cytometry. Division of CD8⁺ T cells was reflected by the decrease of fluorescence intensity. CD8⁺ T cells from BALB/c naïve mice cultured without anti-mouse CD3/anti-CD28/IL-2 were considered the as baseline with minimum proliferation. The average of the triplicate proliferating CD8⁺ T cell wells was recorded.

4.3.9 Murine model of ITP

A murine ITP model was used as previously described (Chow et al., 2010). CD61 KO mice were immunized against CD61⁺ BALB/c platelets and their non-depleted (ND) splenocytes were transferred into SCID mice to initiate ITP (Chow et al., 2010). Splenocytes from some CD61 KO mice were first depleted of CD19⁺ B cells in vitro before transfer to initiate CD8⁺ T
cell-mediated ITP (Bdep in vitro). Other KO mice were B cell depleted (Bdep) in vivo by 2 intravenous injections of an anti-mouse CD20 monoclonal IgG2a antibody (Biogen, Cambridge, MA, USA, 250 ug/mouse) either before or after platelet immunization (to mimic Bdep therapy). CB.17 SCID mice were pre-screened for the presence of IgG by an enzyme-linked immunoabsorbent assay (ELISA) and any mouse with a serum IgG concentration greater than 20μg/mL was deemed “leaky” and excluded. On day -1, the SCID mice were bled via the saphenous vein and pre-treatment platelet counts were measured using a Beckman Coulter Counter-LH750 hematology analyzer. Then, NK cells were depleted by an ip infusion of 50 uL of a rabbit anti-asialo GM1 antibody (Wako Pure Chemical Industries, Ltd). On day 0, the SCID mice were sub-lethally gamma irradiated (2 cGy) and then received an intraperitoneal injection (100 uL) of 1.5 ×10^4 splenocytes from the indicated CD61 KO mice. Some SCID mice received and ip injection of PBS and were negative controls. The peripheral blood of SCID recipients was then collected weekly from the saphenous vein and platelet counts were measured. On day 21, the SCID mice were sacrificed and their lymphoid tissues were used in the indicated assays. Details of the in vitro experimentation and the experimental design are shown in Figure 4.1.

4.3.10 Statistical analysis

Data are expressed as mean±SD, and were analyzed using GraphPad Prism 6.02 software for Windows (GraphPad Software, San Diego, CA).
4.4 Results

4.4.1 Anti-CD20 antibody efficiently depletes B cells and increases the T cell percentages in vivo.

Murine monoclonal anti-CD20 antibodies derived from immunization of CD20 KO mice were first reported by Uchida et al and induce significant B cell depletion in vivo (Uchida et al., 2004a; Uchida et al., 2004b). In our hands, all the anti-CD20 antibody-treated mice were depleted of B cells in the peripheral blood, spleen and mesenteric lymph nodes (Figure 4.2 A and B). In CD61KO mice Bdep before platelet immunization, a significant reduction in IgG anti-platelet antibody production was also observed (Figure 4.2 C and D). Bdep therapy in immune mice significantly increased the percentages of peripheral blood, splenic and lymph node CD3^+CD4^+, CD3^+CD8^+ T cells and FOXP3^+ Treg subpopulations proportionally (Figure 4.3 and 4.4), consistent with previous studies showing that Bdep therapy increases the proportion of non-B cell populations within immune compartments (Yu et al., 2012).

4.4.2 Bdep therapy suppresses CD8+ T cell proliferation in vitro.

B cells have been previously shown to be involved in CD8^+ T cell maintenance and memory cell formation and patients with ITP treated with rituximab have significant alterations of CD8^+ T cells, including increased cytotoxicity of splenic CD8^+ T cells in rituximab non-responders (Audia et al., 2013; Brodie et al., 2008; Shen et al., 2003; Stasi et al., 2007; Thomsen et al., 1996). Therefore we examined the proliferation potential of splenic CD8^+ T cells from non-depleted (ND) or Bdep immune CD61 KO mice. Purified splenic CD8^+ T cells were stained with V450 and cultured with anti-CD3 antibody together with either anti-CD28 antibody and/or recombinant IL-2 for 72 hrs and the fluorescence intensity per cell was analyzed (Figure 4.5 A). Compared with CD8^+ T cells from either naïve BALB/c or ND CD61 immune KO mice, CD8^+ T cells from the Bdep KO mice showed a significantly reduced proliferation upon anti-CD3 and anti-CD28 stimulation (Figure 4.5 B). The deficient CD8^+ T cell proliferation could be rescued by addition of recombinant IL-2 to the cultures (Figure 4.5 C). Further examination of the
splenic CD8+ T cells revealed increased intracellular Granzyme B expression after Bdep therapy (Figure 4.6).

4.4.3 Bdep therapy induces normalization of platelet counts in a murine model of ITP.

We further examined the effect of Bdep therapy on the development of ITP. SCID mice were transferred with splenocytes from immune CD61 KO mice that were either non-depleted (ND) or depleted of CD19+ B cells in vitro to initiate antibody- and T cell-mediated ITP respectively. As previously described (Chow et al., 2010), both splenocyte populations induced significant thrombocytopenia when transferred into SCID mice (Figure 4.7, columns 1-3). In contrast, however, if the CD61 KO mice were Bdep in vivo either before or after platelet immunization, the ability of their splenocytes to induce ITP was completely prevented (Figure 4.7, columns 4-5).
Figure 4.2 Anti-CD20 antibody efficiently depletes B cells in vivo.

A. 

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>Bdep 24h</th>
<th>Bdep Day 27</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220</td>
<td>1.17%</td>
<td>26.68%</td>
<td>2.42%</td>
</tr>
<tr>
<td>CD19</td>
<td>71.72%</td>
<td>0.04%</td>
<td>96.77%</td>
</tr>
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</table>

B. 

C. 

D. 

Titrations
(A) The percentage of CD19⁺B220⁺ B cells (top-right quadrant) in peripheral blood of immune CD61 KO mice without Bdep therapy (ND; left panel), 24 h after first anti-CD20 antibody injection (Bdep 24 h, middle panel), and at the end of the experiment (Bdep Day 27, right panel). (B) The percentage of CD19⁺ B cells in the blood (upper panel), spleen (middle panel) and mesenteric lymph nodes (LN; lower panel) of BALB/c naïve mice (Bc naïve), BALB/c mice depleted of B cells in vivo (Bc Bdep) and CD61 KO mice after CD61⁺ platelet immunization without/with B cell depletion in vivo (KO ND, KO Bdep, respectively). The data shown were at 24 h after anti-CD20 antibody administration. Spleen and mesenteric lymph nodes were taken after sacrifice on day 28. Data were analyzed using one-way ANOVA with Tukey’s post-hoc test. (C) Representative flow cytometric histogram of anti-platelet IgG antibody in immune CD61 KO serum at a dilution factor of 1/400. Top panel was sera from ND and bottom panel was from Bdep pre immune CD61 KO mice (B cell depletion was performed before platelet immunization), with overlays of control prebleed (background) sera depicted in dashed lines. (D) The mean fluorescent intensity (MFI) of individual serum samples from both ND and Bdep immune CD61 KO at serial titrations from 1:200 to 1:1600. Group comparison between ND and Bdep was analyzed using a two-way ANOVA, *P<0.05, *** P<0.001, **** P<0.0001.
Figure 4.3 B cell depletion therapy increases both CD4\(^+\) and CD8\(^+\) T cells in the spleen and lymph nodes.

Spleen (A, C, E) and mesenteric lymph nodes (B, D, F) from mice, treated as indicated in the figure, were examined for CD4\(^+\) and CD8\(^+\) T cells in both total lymphocyte populations (A-D) and CD4/CD8 ratios (E-F). Data were analyzed using a one-way ANOVA with a Tukey’s post hoc test. N=5-12. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure 4.4 The increases of FOXP3$^+$ Tregs were proportional to the increase of CD4$^+$ and CD8$^+$ T cells in the spleen and lymph nodes.

Spleen (A, C, E, G) and mesenteric lymph nodes (B, D, F, H) from indicated mice were examined for CD4$^+$FOXP3$^+$ and CD8$^+$FOXP3$^+$ Tregs in total lymphocyte populations (A-D) and the proportion of FOXP3$^+$ in CD4$^+$ and CD8$^+$ cells, respectively. Data were analyzed using a one-way ANOVA with a Tukey’s post hoc test. N=5-12. *P<0.05, **P<0.01, ****P<0.0001.
Figure 4.5 B cell depletion inhibits CD8+ T cell proliferation in vitro.

A

B

C

anti-CD3/anti-CD28

anti-CD3/IL-2
CD8⁺ T cells were purified from the spleens of BALB/c naïve mice (Bc naïve), platelet immunized CD61 KO mice (KO ND) and platelet immunized CD61 KO mice that received Bdep therapy during immunization (KO Bdep) and stained with the proliferation dye V450 and cultured in vitro with anti-CD3 ± anti-CD28/IL-2 for 72h. (A) Representative flow cytometric dot plot analysis of CD8⁺ T cell proliferation when either not stimulated (top panels) or stimulated with anti-CD3/CD28 antibodies for 72 hours. A cell division cycle is characterized by sequential halving of the V450 fluorescence. Cumulative data of (B) CD8⁺ splenic T cells stimulated with anti-CD3/anti-CD28 and (C) anti-CD3/IL-2. Data in B and C are expressed as the mean ± SD of percent CD8⁺ T cells proliferating. Data were analyzed using one-way ANOVA with a Tukey’s post-hoc test (N=5-8. ***P<0.001, ****P<0.0001, NS, non-significant).
Figure 4.6 B cell depletion therapy increases Granzyme B expression levels within CD3⁺CD8⁺ T cells.

CD3⁺CD8⁺ T cells in the spleen of the indicated mice were examined for Granzyme B, IFN-γ and CD44 expression levels. Data were analyzed using one-way ANOVA with a Tukey’s post hoc test. N=5-9. **P<0.01.
Figure 4.7 In vivo B cell depletion results in a normalization of platelet counts in a murine model of T cell-mediated ITP.

Platelet counts in transferred recipient SCID mice after 21 days post engraftment of $3 \times 10^4$ splenocytes. SCID mice were either transferred with PBS or with non-depleted splenocytes from platelet-immunized CD61 KO mice (ND), splenocytes from ND KO mice but depleted of CD19$^+$ B cells in vitro (ND Bdep in vitro) or splenocytes from platelet-immunized CD61 KO mice depleted in vivo with anti-CD20 antibody before platelet immunization (B cell dep in vivo, pre) or after platelet immunization (B cell dep in vivo, post). Each data point represents one SCID mouse. Data were analyzed using one-way ANOVA with a Tukey’s post test (*$P<0.05$, ***$P<0.001$).
4.5 Discussion

In our current study, anti-CD20 therapy significantly depleted B cells in vivo, and suppressed CD8$^+$ T cell proliferation in vitro in an IL-2 dependent way. Anti-CD20 therapy lead to a rescue of CD8$^+$ T cell-mediated ITP in established murine model, which suggests an alternative explanation independent of suppression of antibody-mediated ITP for the therapeutic effect of rituximab for patients with ITP.

The increased the percentages of peripheral blood, splenic and lymph node CD3$^+$CD4$, CD3^+CD8^+ T cells and FOXP3$^+$ Treg subpopulations after anti-CD20 therapy was proportional (Figures 4.3, 4.4). This is consistent with previous studies showing that Bdep therapy increases the proportion of non-B cell populations within the immune compartments (Yu et al., 2012).

We found that CD8$^+$ T cells purified from Bdep immune CD61 splenocytes had impaired proliferation as compared to CD8$^+$ T cells from non-depleted mice, which could be rescued by addition of recombinant IL-2 to the cultures (Figure 4.5 C). This could be explained by at least two different mechanisms. First, B cells may serve as APCs and present self platelet antigens to CD4$^+$ and CD8$^+$ T cells in a proinflammatory manner and promote the CD8$^+$ T cell mediated ITP (Kambayashi and Laufer, 2014). The removal of B cells by anti-CD20 antibody thus dampens the stimulation of T cell responses. Second, CD20$^{dim/-}$ regulatory B cells (Bregs) may be upregulated after B cell depletion therapy and play an important role in the suppression of CD8$^+$ T cell mediated ITP. For example, an increased number of CD20$^{dim}$ regulatory B cells (Bregs) have been shown capable of suppressing CD4$^+$ and CD8$^+$ T cell proliferation in vitro (Bodogai et al., 2013; Lee-Chang et al., 2014). Using the experimental autoimmune encephalomyelitis (EAE) mouse model, Shen et al showed an important role of IL-35 producing and IL-10 producing plasma B cells which population are usually negative for CD20$, in the suppression of autoimmune T cell responses (Reff et al., 1994; Shen et al., 2014). In our current study, further examination of the splenic CD8$^+$ T cells revealed increased intracellular Granzyme B expression after Bdep therapy (Figure 4.6 A). This may be related to how CD20$^{low}$-1BBL$^+$ B regulatory cells regulate Granzyme B expression in CD8$^+$ T cells (Bodogai
et al., 2013; Lee-Chang et al., 2014). Although Granzyme B expression in CD8+ T cells was found to be increased in follicular lymphoma patients at diagnosis, which was associated with better prognosis, it appeared unchanged in patients with ITP (Audia et al., 2013; Laurent et al., 2011). Taken together, our results suggest that in vivo Bdep therapy interferes with in vitro IL-2-dependent CD8+ T cell activation but more research is required to characterize how this phenomenon occurs.

We further examined the effect of Bdep therapy on the development of ITP. When the CD61 KO mice were Bdep in vivo either before or after platelet immunization, we found that the ability of their splenocytes to induce ITP was completely prevented.

Mechanistically, Bdep therapy in vivo may actively suppress or exhaust the proliferative potential of pathogenic CD8+ T cells upon activation and thereby limit their ability to induce ITP. Support for this possibility comes from studies showing that T cells, in the absence of B cells, have proliferation and memory development defects (Brodie et al., 2008; Shen et al., 2003; Thomsen et al., 1996). In addition, although B cell depletion in vivo may modulate the balance between pro- and anti-inflammatory T cell-derived cytokines (Stasi et al., 2007), we did not observe any significant differences in IFN-γ production by CD8+ T cells (Figure 4.6 B) or intracellular expression of IFN-γ/IL-4 in CD4+ T cells (N=5-8, data not shown) after Bdep therapy. Furthermore, in accordance with previous studies, the activation marker CD44 was not increased on the CD8+ T cells after Bdep therapy (Figure 4.6 C) (Brodie et al., 2008; Shen et al., 2003; Xiu et al., 2008). Of interest, interruption of the B and T cell interactions by, for example, CD40L antibody, shows a similar therapeutic effect as rituximab in ITP suggesting a direct interaction between B cells and T cells is essential for ITP induction (Cooper et al., 2012). More studies are required, however, to further elucidate these mechanisms.

In summary, our study suggests that the effectiveness of anti-CD20 therapy is due to induction of a significant CD8+ T cell activation/proliferation defect via IL-2 blockade that correlates with their inability to induce thrombocytopenia. This may provide an additional explanation for the therapeutic effects of rituximab in T cell-mediated ITP.
Chapter 5

Discussion And Future Directions
5.1 Discussion

This thesis aimed to characterize the effects of different therapies on antibody-mediated and T cell-mediated immune thrombocytopenia (ITP) separately in murine models. Current ITP treatment options are not satisfactory due to adverse reactions, low long-term response rates and costs (Provan et al., 2010). Understanding the pathogenesis of the disease is essential to the future development of effective and safe therapies. Understanding the mechanisms of current therapies could not only broaden our knowledge of the pathogenesis of the disease but also help us discover new therapies. Animal models of ITP are useful tools that allow us to examine the pathogenesis of the disease and action mechanisms of the therapies.

To achieve my goals, first, I studied the effects of different anti-platelet antibodies on megakaryocytes in a passive ITP model and the impact of IVIg therapy. Most of the anti-platelet antibodies did not significantly change the number or morphology of megakaryocytes and neither did IVIg therapy, suggesting limited effects on megakaryocytes. Second, I evaluated the effectiveness of allogeneic platelet transfusion therapy and its effect on megakaryocytes in ITP using the laboratory’s active murine ITP model. I found an inhibitory effect of the transfusion therapy on T cell-mediated ITP associated with ameliorated megakaryocyte abnormalities. I further characterized the interaction between allogeneic platelets and T cells in vivo and in vitro. Third, I investigated the interaction between B cells and T cells in ITP by examining T cell-mediated ITP development after B cell depletion therapy and found that T cell-mediated ITP was abolished without B cells and this was associated with a decreased proliferation of the CD8^+ T cells.

Different antibodies have different effects on megakaryocytes

Megakaryocyte abnormalities in ITP were first reported by Dameshek et al in 1946, although the reason was not clear at that time. Anti-platelet antibodies were the first immune
factors discovered to be responsible for the pathogenesis of ITP and these studies dated back to the 1950s-1960s (Harrington et al., 1951; Shulman et al., 1965). Currently, it is known that about two thirds of patients with ITP have anti-platelet antibodies that primarily target platelet GPIIbIIIa and GPIb molecules (Chan et al., 2003; Karpatkin et al., 1972). However, the effect of anti-platelet antibodies on megakaryocytes was not linked together in the pathogenesis of ITP until the recognition that megakaryocytes and platelets express all the platelet lineage markers (Michelson, 2013). In the last decade, using cultured megakaryocytes derived from hematopoietic stem cells in vitro, it has been shown that both anti-platelet serum and T cells from patients with ITP could inhibit megakaryocyte proliferation and maturation in vitro, as indicated by the number, size, ploidy and apoptosis of the megakaryocytes and the number of proplatelets/platelets produced by megakaryocytes (Balduini et al., 2008; Chang et al., 2003; Li et al., 2007; McMillan et al., 2004). Since patients with ITP have both B and T cell abnormalities, the effect of the antibodies independent of T cells on megakaryocytes in vivo is unknown.

In our study, we fulfilled **Aim 1** by examining the effects of various anti-platelet antibodies on megakaryocytes in a 24 h passive ITP model. We used both anti-platelet sera which contained polyclonal antibodies, and monoclonal antibodies with either GPIIbIIIa or GPIb specificities. Although all the sera and antibodies induced significant thrombocytopenia in mice (passive ITP) within 24 h, only an anti-GPIIIa serum and an anti-GPIIb monoclonal antibody (MWReg30) significantly increased the megakaryocyte counts. These data suggest that most anti-platelet antibodies with different specificities significantly affect platelet counts, presumably through peripheral splenic destruction, but few affect megakaryocyte counts or morphology. However, further studies are needed to confirm this. In addition, we evaluated the therapeutic effect of IVIg on ITP induced by these different antibodies and on megakaryocytes in vivo. In the majority of passive ITP groups, although IVIg could significantly ameliorate thrombocytopenia, it did not significantly change the number of megakaryocytes as compared with non-treated control groups. This suggests that IVIg may not directly regulate the thrombopoiesis in ITP.
In this study, due to limited time and resources, there are several experiments that we have planned but have not carried out. Previous electron microscopy (EM) studies in patients with ITP have shown abnormal apoptotic changes in megakaryocytes such as cytoplasmic vacuolization, distended DMS and condensed chromatin (Houwerzijl et al., 2004). Besides the EM study findings, there is also evidence of antibody-induced increased apoptosis of cultured human megakaryocytes in vitro (Balduini et al., 2008; Chang et al., 2003; Li et al., 2007; McMillan et al., 2004). We also need to examine the ultrastructure of the megakaryocytes in the passive ITP model by EM, and examine the apoptotic markers of the megakaryocytes using immunohistochemistry staining or flow cytometry techniques. Moreover, our lab has recently developed a protocol to isolate and culture primary megakaryocytes from mouse bone marrow. Thus, in combination with other techniques just mentioned, I could further examine the direct effect of antibodies on megakaryocytes and whether the antibodies trigger the thrombopoiesis signaling pathways of megakaryocytes in vitro. Nonetheless, it appears from this chapter that while most anti-platelet antibodies can induce thrombocytopenia independently of T cells, they generally fail to induce changes in megakaryocyte numbers or morphology in the bone marrow. This may suggest that megakaryocyte abnormalities observed in ITP may be due primarily to T cells and I attempted to address this in the following chapter.

**Allogeneic platelet transfusions benefit patients with T cell-mediated ITP**

Historically, allogeneic platelet transfusions were not recommended for the management of ITP (Ahn and Harrington, 1977; Slichter, 1980; Sprague et al., 1952). However, some clinical evidence emerged to challenge this dogma by showing that allogeneic platelet transfusions might in fact benefit patients with ITP (Carr et al., 1986; Spahr and Rodgers, 2008). However, to date, there is still a lack of substantial investigations on this subject.

Allogeneic platelets have been shown to have transfusion-related immunomodulation (TRIM) effects and our previous work has found significantly improved allogeneic skin graft survival after allogeneic platelet transfusions (Aslam et al., 2008). We found this TRIM effect was associated with the MHC class I molecules on platelets. MHC class I on platelets is
denatured and lacks β2 microglobulin when compared to MHC class I on nucleated cells. We and others have shown that allogeneic platelets cannot stimulate CD8⁺ T cell responses (Gouttefangeas et al., 2000; Kao, 1987; Kao et al., 1986; Semple et al., 1995). Moreover, the immune functions of transfused platelets could also potentially regulate the immune responses in the recipients, for example by releasing proinflammatory or anti-inflammatory cytokines (Kapur et al., 2015b).

We examined whether this TRIM effect would affect antibody- or T cell-mediated platelet and megakaryocyte destruction in our active ITP mouse model. We fulfilled Aim 2 by showing that while allogeneic platelet transfusions did not affect antibody-mediated ITP, they completely prevented the development of T cell-mediated ITP. Both T cell-mediated platelet destruction in the spleen and increased megakaryocyte number in the bone marrow were normalized by allogeneic platelet transfusions. Since CD8⁺ CTLs are recognized as the major cell population responsible for T cell-mediated ITP in human, then we wondered: could this TRIM effect be related to an inhibition of CTL cytotoxicity? In vitro cytotoxicity function assays were carried out to address this question. In an ideal scenario, to test anti-CD61 specific cytotoxicity, target cells that express CD61 peptide through intact syngeneic MHC class I complexes should be used. However, we could not find target cells that met the criteria. None of the cell-lines with syngeneic MHC class I were CD61 positive. Therefore we picked a macrophage cell-line PU5-1.8 and pulsed them with CD61⁺ platelets, and used these cells as target cells after confirming that they became weakly CD61 positive. Subsequent cytotoxicity assays showed increased anti-CD61 T cell-mediated cytotoxicity in immune CD61 KO mice and SCID adoptively transferred with immune splenocytes. In contrast, CD61 mice transfused with allogeneic CD61⁺ platelets showed no cytotoxicity against target cells. We further cultured immunized CD61 KO splenocytes in vitro together with either syngeneic or allogeneic CD61⁺ platelets and the latter incubations consistently showed a significantly reduced cytotoxicity level. This suggests that allogeneic platelets inhibit the anti-CD61 specific CTL responses (Aim 2). Furthermore, weekly allogeneic platelet transfusions rescued pre-existing T cell-mediated ITP, suggesting a therapeutic effect of allogeneic platelet transfusions. Based on these observations, we concluded
that ‘the cell-mediated ITP could be abolished by transfusions of allogeneic platelet MHC I antigens’, ‘indicating a potential immunomodulatory benefit of allogeneic platelet transfusions in ameliorating T cell-mediated ITP’ (Guo et al., 2014). Of interest, there was significant increases in megakaryocyte numbers and abnormal morphology in the SCID mice with active T cell-mediated ITP and these abnormalities were abolished by the allogeneic platelet transfusions. These results support our claim in Chapter 2 that antibodies alone do not necessarily induce megakaryocyte abnormalities but only when in conjunction with anti-platelet CTL’s are megakaryocyte abnormalities observed.

A concern in this study is MHC class I polymorphisms in different mouse strains. In other words, are the allogeneic platelet transfusion effects only seen when H-2\(^b\) platelets are transfused into H-2\(^d\) recipients. In our current study, most of the work shown was carried out in BALB/c (H-2\(^d\)) mice. To exclude the possibility that the TRIM effects observed were not H-2\(^d\) specific, we replicated the same experiments in reverse where C57BL/6 CD61 KO mice (H-2\(^b\)) were used as transfusion recipients of BALB/c (H-2\(^d\)) platelets. Thus it appears that our transfusion effect holds for different mouse strains with MHC incompatibilities.

Although we found that allogeneic platelet transfusions could inhibit the anti-CD61 specific T cell-mediated ITP, we did not further examine whether this was due to a direct interaction between allogeneic platelets and CTLs, or an indirect effect with the involvement of other cells such as antigen presenting cells. Overall, the novel finding that allogeneic platelet transfusions have an inhibitory effect on T cell-mediated ITP provides an explanation for the observation of continuous increments in platelet counts after cessation of platelet transfusions in patients with ITP, and supports the usage of allogeneic platelet transfusions in the management of patients with ITP.

**B cells help CD8\(^+\) T cell responses in ITP**

Since the initial success of raising platelet counts of patients with ITP using rituximab, there has been an increasing interest in understanding the mechanisms of rituximab in ameliorating the disease (Stasi et al., 2001). The initial rationale was that rituximab inhibits
antibody-mediated platelet destruction by removal of B cells. However, anti-platelet antibody titers did not necessarily decrease in responders and anti-platelet antibody-negative patients could also respond to the therapy suggesting antibody-independent mechanism(s) may exist (Cooper et al., 2012). The effectiveness of rituximab in other CD8\(^+\) T cell mediated diseases such as diabetes also suggests immunomodulatory effects of B cell depletion therapy on T cell responses (Hu et al., 2007). Therefore we hypothesized that B cell depletion therapy could regulate CD8\(^+\) T cell-mediated ITP.

B cells could regulate CD8\(^+\) T cell-mediated immune responses in several ways. An antigen specific CD8\(^+\) T cell immune response could be divided into at least three different stages: a) the initial activation, b) clonal expansion (extensive proliferation) and antigen clearance, and c) apoptosis of most effector cells and differentiation of a small population into memory CD8\(^+\) T cells (Jenkins, 2013). The CD8\(^+\) T cell responses are regulated by APCs, CD4\(^+\) helper T cells and cytokine patterns (Fooksman, 2014; Jenkins, 2013; Rosette et al., 2001). In the first stage, B cells do not seem to play an important role. This is supported by the observation that in B cell deficient mice or B cell depleted mice, antigen specific CD8\(^+\) T cell immune response could be elicited (Lykken et al., 2014; Thomsen et al., 1996). In our study, the proportion of CD8\(^+\) T cells in the total T cell population was also comparable between B cell depleted and non-depleted immune CD61 KO mice. However, B cell depletion may regulate the proliferation of CD8\(^+\) T cells, as suggested by our CD8\(^+\) T cell proliferation in vitro experiment. Consistently, Lykken et al also showed decreased CD8\(^+\) T cell number 7 days after initial LCMV infection in B cell depleted mice (Lykken et al., 2014). Moreover Thomsen et al found impaired CD8\(^+\) T cell responses in B cell deficient mice after chronic LCMV infection (Thomsen et al., 1996). In addition, B cell deficiency may also impair the formation of CD8\(^+\) T cell memory. This was proposed by Thomsen et al as they did not find a CD8\(^+\) T cell memory response upon secondary antigen stimulation (Thomsen et al., 1996). Later Shen et al confirmed a positive role for B cells in CD8\(^+\) T cell memory formation (Shen et al., 2003). In addition to the direct effect on CD8\(^+\) T cell responses, B cell depletion could also indirectly regulate the CD8\(^+\) T cell responses through, for example, downregulation of CD4\(^+\) helper T cells and
upregulation of Tregs (Lund and Randall, 2010). Cytokines produced by B cells may also impact the CD8$^+$ T cell responses, for example IL-2, IL-10, IL-6 and IFN-γ (Lund, 2008).

In 2007, Stasi et al reported normalized Th1/Th2 (IFN-γ$^+$/IL-4$^+$ of CD4$^+$ T cells) and Tc1/Tc2 (IFN-γ$^+$/IL-4$^+$ of CD8$^+$ T cells) ratios in the peripheral blood of rituximab responders but not in patients with active ITP nor in rituximab non-responders suggesting an immunomodulatory effect of rituximab on both CD4$^+$ and CD8$^+$ T cell responses (Stasi et al., 2007). Similarly, Nazi et al reported a decreased number of IFN-γ$^+$-producing T cells in patients with ITP upon exogenous antigen stimulation after rituximab administration (Nazi et al., 2013). Subsequently, further examination of the CD4$^+$ T cells showed restored number and function of CD4$^+$ T regulatory cells (Tregs) after rituximab therapy which may help explain the restored balance within CD4$^+$ and CD8$^+$ T cells (Stasi et al., 2008). In contrast with rituximab responders, spleens from patients who failed rituximab therapy still showed decreased Tregs and increased memory CD8$^+$ T cells (Audia et al., 2011; Audia et al., 2013).

Beyond the changes in numbers and frequencies of different cell populations, our knowledge of how B cell depletion therapy can regulate CD4$^+$ and CD8$^+$ T cell responses and restore Tregs in ITP still remains a matter of debate. One limitation is the impossibility of spleen sample acquisition from responder patients for more detailed studies. In addition, although it is well accepted that in patients with ITP, there are both antibody-mediated and CD8$^+$ T cell-mediated mechanisms, it has been difficult to sort out the exact contributions of one pathway over the other.

In Chapter 4, our murine active ITP model was used for the study of the B cell depletion therapy in T cell-mediated ITP. We initially tested a number of commercially available anti-CD20 mAb but none of them caused complete depletion of B cells in vivo. We obtained a novel anti-mouse CD20 mAb from Biogen and this antibody proved to be an almost total depletion antibody and we used this antibody for our Chapter 4 studies.

We examined the B cell percentages in the blood, spleen, and mesenchymic lymph nodes, as well as determination of the anti-platelet antibody titers in the blood (Aim 3). The anti-mouse
CD20 IgG2a antibody used in our study successfully depleted 80-99% of the B cells in vivo. Next, we established a mouse model of B cell depletion therapy in CD8⁺ T cell-mediated ITP using this antibody (Aim 3). By collecting immune splenocytes from CD61 KO mice, depleting the splenic B cells in vitro and then transferring the depleted cells into SCID mice, CD8⁺ T cell-mediated ITP independent of B cells could be induced (Chow et al., 2010). To further fulfill Aim 3, the anti-CD20 mAb was administrated into the CD61 KO mice before or after platelet immunization (Bdep pre and post respectively), followed by transferring their splenocytes into SCID mice. Analyzing the platelet counts in SCID mice showed that B cell depletion therapy, whether before or after platelet immunization, significantly ameliorated the T cell-mediated ITP. We further investigated potential mechanisms through which B cells could regulate the CD8⁺ T cell-mediated ITP. The CD8⁺ T cell proliferation assay in vitro revealed that CD8⁺ T cells isolated from the immune CD61 KO spleens without B cells proliferated significantly less compared with T cells from intact (non-depleted) spleens and this appeared to be due to a lack of IL-2 production. These data suggest an important role of B cells for CD8⁺ T cell immune responses upon immune challenges and depletion of B cells leads to an impaired CD8⁺ T cell response which could no longer elicit thrombocytopenia in our mice. This may provide a novel explanation for the antibody-independent therapeutic effect of rituximab in humans.

To better understand the role of B cells on CD8⁺ T cell immune responses in ITP, we further characterized the CD8⁺ T cells from the immune CD61 KO mice treated with B cell depletion therapy by flow cytometry, including the CD4/CD8 T cell ratio, percentages of CD8⁺ Tregs, the granzyme B, IFN-γ and CD44 expression levels. We only observed significantly increased expression of granzyme B with B cell depletion therapy, indicating that the regulation of CD8⁺ T cell immune responses by B cells are quite specific and probably not due to any broad secondary responses.

Although the anti-CD20 antibody in our study successfully depleted most of the B cells in vivo, there were still a small population of B cells remaining in the spleen and lymph nodes. Theoretically, these residual B cells might still be able to mediate some effects on the T cells. To better address whether the ameliorated CD8⁺ T cell-mediated ITP is due to the removal of
the majority of B cells, or due to the modulation of residual B cells by anti-CD20 antibody, experiments could be carried out using pepsin digested anti-CD20 antibody instead of intact antibody. Pepsin cuts the antibody into F(ab')\textsubscript{2} fragments (Murphy et al., 2012; Paul, 2013) that can still bind to B cells but would not be able to deplete B cells in vivo through Fc-γ receptor mediated phagocytosis or ADCC. In this way, comparison of the immune CD61 KO mice that received pepsin digested or intact antibody will reveal the potential regulatory effect, if there are any, by residual anti-CD20 coated B cells on the CD8\textsuperscript{+} T cell-mediated ITP.

Overall, the inhibition of CD8\textsuperscript{+} T cell proliferation and prevention of the development of ITP by a mouse anti-CD20 mAb suggest a novel role for B cells in maintaining CD8\textsuperscript{+} T cell responses. This provides us with another explanation for the therapeutic effect of B cell depletion therapy for ITP and potentially for other T cell-mediated autoimmune diseases as well.

**Animal models of ITP are useful tools with limitations**

There are several critical challenges in human studies on ITP. First of all, due to ethical reasons, access to immune organs and tissues is restricted. For example, while spleen is the major lymphoid site for adaptive immune responses against platelets to initiate, it is impossible to examine and compare spleens from treatment responders (not splenectomized) versus nonresponders. Second, although it is clear that both B cells and T cells are involved in the pathogenesis of the disease, their relationship in ITP remains ambiguous. Patients with detectable autoreactive anti-platelet antibodies are usually considered to possess B cell dominant pathological immune responses and patients without are considered as T cell dominant. However, patients may actually have both B cells and T cells that mediated thrombocytopenia in vivo and it is difficult to examine the individual roles of B cells or T cells separately. Third, although the studies by Harrington made fundamental discoveries, transfer of potential pathological components from patients with ITP into healthy volunteers are not ethical. In addition, clinical trials of new therapies are restrictively regulated and the recruitment of adequate number of patients is a challenge considering the low incidence of the disease.
In contrast, animal models of ITP are useful tools that can easily overcome the limitations mentioned above and allow us to further understand the disease and the therapies. Using the passive and active ITP mouse models, we were able to examine the role of anti-platelet B cells and T cells separately. For instance, in our active ITP model, by transferring either B cell depleted or CD8⁺ T cell depleted splenocytes from the immune CD61 KO into SCID mice, we could examine the effect of allogeneic platelet transfusions on B cell-mediated ITP and T cell-mediated ITP separately (Guo et al., 2014). Examination of the T cells in B cell depletion therapy treated mice also revealed a novel mechanism of the anti-CD20 antibody - the decreased CD8⁺ T cell proliferation after B cell depletion therapy, which could help to explain the antibody-independent therapeutic effect of rituximab in patients (Guo et al., 2016). In our previous work, we also found abnormal Treg development in the thymus which may contribute to the pathogenesis of ITP but remains unclear in humans with ITP (Aslam et al., 2012).

There are, however, limitations with animal studies. Firstly, our findings in mice may not necessarily represent the situation in humans. In our active ITP mouse model, the thrombocytopenia in SCID mice was induced by anti-CD61 (GPIIIa) specific immune responses. We choose CD61 KO mice because anti-GPIIbIIIa antibodies are the most frequent detectable antibodies in patients with ITP. However, most patients have polyvalent autoreactive antibodies that may be reactive with multiple receptors on platelets (Liel et al., 2014; van Leeuwen et al., 1982). Secondly, in our study, B cell depletion therapy significantly alleviated T cell-mediated ITP, providing a novel explanation of antibody-independent mechanism of the therapy. In humans, however, only some of the anti-platelet antibody negative patients respond to B cell depletion therapy, indicating a more complex situation in patients than in our mouse model (Cooper et al., 2012).
5.2 Conclusion

I examined both antibody-mediated and T cell-mediated ITP in our mouse models and evaluated three different treatments for ITP including IVIg, allogeneic platelet transfusions and B cell depletion therapy. I found that IVIg could inhibit antibody-mediated thrombocytopenia but did not necessarily affect megakaryocytes in the bone marrow. In contrast, allogeneic platelet transfusions significantly inhibited both T cell-mediated thrombocytopenia and bone marrow MK abnormalities. Similar findings were also observed with B cell depletion therapy in its ability to rescue T cell-mediated ITP through inhibiting CD8+ T cell proliferation.

In general, I was able to address my primary research questions and achieved my goals. Using the passive and active ITP mouse models, I discovered that megakaryocytes may be resistant to the effects of antibodies in ITP and novel immunomodulatory mechanisms of both allogeneic platelet transfusion therapy and B cell depletion therapy exist in ITP. My work has challenged some prevailing theories, one of which believes that antibodies not only destroy platelets peripherally but also inhibit platelet production in the bone marrow and another of which thinks that platelet transfusions are effective as an ITP treatment. Nonetheless, I am still cautious of my claims due to the limitations of animal studies and of potential discrepancies between my findings and those in human ITP. It is my hope that my work can lay a knowledge foundation for the development of new therapies that may be curative and enable patients to maintain a good quality of life.
5.3 Future Directions

Megakaryocyte morphology and viability evaluations in the antibody-mediated passive ITP model

In Chapter 2, I studied the number and morphology of megakaryocytes with H&E staining and found discrepant effects of the different antibodies. In particular, I found increased numbers of megakaryocytes in ITP mice which were induced by MWReg30, an anti-CD41 (anti-GPIIb) antibody. The rest of the monoclonal antibodies did not seem to affect the megakaryocytes number nor morphology. Our in vivo findings contradicted several in vitro studies on cultured human megakaryocytes that showed anti-platelet antibodies from patients with ITP significantly increased megakaryocyte apoptosis growth in vitro (Balduini et al., 2008; Chang et al., 2003; Li et al., 2007; McMillan et al., 2004). Thus, to better address the effect of anti-platelet antibodies on megakaryocytes in vivo, further morphology as well as viability analyses of megakaryocytes are planned using the passive ITP model. For example, 24 hours after injection of anti-platelet antibodies, the mice will be sacrificed and their femurs collected for the following experiments. First, to evaluate whether the anti-platelet antibodies affect megakaryocyte integrity and intracellular structures, bone marrow will be collected and megakaryocytes will be separated from the bone marrow and processed for EM examinations. Second, to clarify whether anti-platelet antibodies induce megakaryocyte apoptosis in 24h in vivo, the expression level of caspase-3 and annexin V will be examined in the megakaryocytes by flow cytometry and fluorescence microscopy (Josefsson et al., 2011; Lev et al., 2014; Leytin et al., 2006; Li et al., 2007).

The effect of MWReg 30 on megakaryocytes

In Chapter 2, we showed significantly increased numbers of megakaryocytes in the bone marrow in MWReg30 treated mice within 24 hours. The total megakaryocyte number calculations in our study included both mature megakaryocytes and immature megakaryocytes including promegakaryoblasts, megakaryoblasts and promegakaryocytes. It is not clear whether MWReg30 stimulated the increase of megakaryocytes at different stages proportionally or
mainly regulated megakaryocytes at a certain stage. One way to separate megakaryocytes of different stages is by their ploidy, which increases from 2N to up to 32N through maturation (Italiano Jr. and Hartwig, 2013). Therefore **we will examine the ploidy of megakaryocytes from the passive ITP mice using an intracellular nucleic acid stain propidium iodide followed by flow cytometry and fluorescence microscopy analysis.** In addition, although all the monoclonal antibodies we used in Chapter 2 could induce thrombocytopenia, MWReg30 showed a distinctive in vivo effect on megakaryocytes. It is possible that MWReg30 triggers signaling pathways which upregulate megakaryocyte maturation and proliferation. One of the most important pathways in megakaryocyte maturation and proliferation is the JAK/STAT pathway (Pallard et al., 1995; Sattler et al., 1995). **We are planning to examine whether the binding of MWReg30 to megakaryocytes activates the JAK/STAT pathway** by examining the transcription levels of JAK2 and STAT5 mRNAs and the phosphorylation of JAK2 and STAT5 proteins.

**A potential role of megakaryocytes in triggering CD8\(^+\) T cell immune responses in ITP**

In the literature, most of the studies on megakaryocytes and CD8\(^+\) T cells in ITP have being focusing on the pathological role of autoreactive CD8\(^+\) T cells in mediating megakaryocyte destruction (Chang et al., 2003; Li et al., 2007; McMillan et al., 2004; Olsson et al., 2003). As mentioned in Chapter 1, both megakaryocytes and platelets have immunomodulatory roles and dysfunction of megakaryocytes and platelets may lead to altered immunity. Recently, some preliminary work of my colleagues suggests that megakaryocytes can function as potent APCs that can directly activate antigen specific CD8\(^+\) T cell responses. Thus, **we hypothesize that megakaryocytes may not only be the target of immune T cells in ITP but also an initiator of the autoreactive CD8\(^+\) T cell responses in ITP.** We would test our hypothesis by pulsing megakaryocytes from BALB/c CD61 KO mice with syngeneic WT platelets in vitro and then culture them with naïve or immune CD8\(^+\) T cells to examine whether megakaryocytes can stimulate anti-CD61 CD8\(^+\) T cell immune responses. The CD8\(^+\) T cell responses would be monitored by intracellular IL-2 and IFN-γ production and the proliferation and cytotoxicity assays in vitro.
The therapeutic potential of allogeneic platelet transfusions in patients with ITP

In Chapter 3, we found an inhibitory effect from allogeneic platelet transfusions on T cell-mediated ITP. With the evidence that allogeneic MHC class I molecules on platelets are denatured and disable the CD8+ T cell mediated platelet destruction, we look forward to continuing our studies using human samples. **Whether human allogeneic platelets could inhibit the CD8+ T cell mediated platelet destruction and CD8+ T cell proliferation in vitro will be examined.** Patients with ITP as well as healthy volunteers will be recruited and their peripheral blood will be collected and CD8+ T cells will be isolated. The cytotoxic activity of the CD8+ T cells from patients with ITP or healthy volunteers will be evaluated by first incubating the CD8+ T cells with autologous or allogeneic platelets and then examining the apoptosis level of platelets. The proliferation potential of the human CD8+ T cells in vitro will be examined similarly as in our mouse studies in Chapter 4. The isolated CD8+ T cells will be labeled with the fluorescent Violet Proliferation Dye 450 (V450) and cultured with either autologous or allogeneic platelets, and with anti-human CD3 antibody/anti-human CD28/recombinant IL-2 stimulation. The proliferation of CD8+ T cells will be analyzed after 72h by flow cytometry.

**Characterization of how B cells regulate CD8+ T cell responses**

In Chapter 4, we found decreased CD8+ T cell proliferation after B cell depletion therapy. Although there is evidence of impaired CD8+ T responses in B cell deficient or depleted mice, our study provides the first mechanistic explanation (Brodie et al., 2008; Shen et al., 2003; Thomsen et al., 1996). Further studies on how B cells promote CD8+ T cell proliferation in vivo may not only improve our understanding of ITP as well as other T cell-mediated immune responses, but may also provide potential novel therapeutic strategies for T cell-dominant diseases. **We can further characterize the CD8+ T cells including the expression level of surface receptors, intracellular cytokines and transcription factors that are involved in CD8+ T cell proliferation and memory T cell formation**, such as CD28, CTLA-4, CD40L.
programmed death-1 receptors, IL-2, activation and nuclear localization of nuclear factor NFκB, CCR7, CD62L.

The development of thymic regulatory T cells (Tregs) in our mouse model

In addition, in our active ITP mouse model, we found decreased FOXP3+ Tregs in the periphery, associated with an increase of Tregs in the thymus (Aslam et al., 2012). We also found an increase of Tregs in the periphery with the amelioration of the disease after treatment (data not show). We need to further study the potential mechanisms responsible for the retention of Tregs in the thymus during ITP, and potential strategies to promote their release from the thymus as a future treatment for ITP.

The role of natural killer (NK) cells in ITP

As mentioned in Chapter 1, one mechanism of the antibody induced immunological destruction is through the antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells (Paul, 2013). However, in the case of ITP, discrepant findings have been documented concerning whether NK cells are activated and involved in the pathogenesis of ITP (Breunis et al., 2008; Engelhard et al., 1986; Garcia-Suarez et al., 1993; Semple et al., 1991; Semple and Freedman, 1994). This question has not been well addressed so far and the role of NK cells in ITP remains mysterious. We will examine whether NK cells are involved in ITP using our active ITP mouse model. We will first deplete NK cells in BALB/c CD61 KO mice in vivo using the anti-Asialo GM1 antibody on the day prior to initial platelet immunization. Rabbit immunoglobulin will be used as an isotype control. On the following day, CD61 KO mice in both groups will be immunized with syngeneic WT platelets and continue to be immunized weekly. The efficiency of NK cell depletion will be further examined by detecting the percentage of CD56+CD3– NK cells in peripheral blood weekly and in the spleen and lymph nodes at week 4. After four weeks, immune CD61 KO mice will be sacrificed and their splenocytes will be further transferred either as non-depleted or in vitro CD56+CD3– NK cell depleted into SCID mice at a dose of 3×10⁴ cells/mouse and the platelet counts of the SCID
recipient mice will be monitored. This will allow for an initial analysis of whether NK cells play a role in murine ITP.

We hope the outlined experiments will not only improve our understanding of ITP, but also shed light on new therapeutic strategies with better targeted action and less side effects culminating in a more individualized therapeutic approach to ITP.
REFERENCES


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Cardarelli, P.M., Quinn, M., Buckman, D., Fang, Y., Colcher, D., King, D.J., Bebbington, C., and Yarranton, G. (2002). Binding to CD20 by anti-B1 antibody or F(ab')(2) is sufficient for induction of apoptosis in B-cell lines. Cancer immunology, immunotherapy : CII 51, 15-24.


induced by antibody- and CD8+ T cell-mediated responses that are differentially sensitive to therapy. Blood 115, 1247-1253.


cells impacts the regulation of T-cell immunity and homeostasis in vivo. Proceedings of the National Academy of Sciences of the United States of America 109, 11288-11293.


intrinsic apoptosis pathway that must be restrained to survive and produce platelets. The Journal of experimental medicine 208, 2017-2031.


thrombocytopenia: systematic review. BJOG : an international journal of obstetrics and gynaecology 117, 1335-1343.


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formation contribute to decreased platelet count during acute simian immunodeficiency virus infection in pig-tailed macaques. The Journal of infectious diseases 208, 874-883.


Robb-Smith, A.H. (1967). Why the platelets were discovered. British journal of haematology 13, 618-637.


Functional platelet defects in children with severe chronic ITP as tested with 2 novel assays applicable for low platelet counts. Blood 123, 1556-1563.


APPENDICES


<table>
<thead>
<tr>
<th>Type of bleeding</th>
<th>Definition</th>
<th>Grade based on the worst incident episode since last visit</th>
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<tbody>
<tr>
<td>Skin(epidermis and dermis)</td>
<td>Red (recent) or purplish (a few days old) discoloration in the skin with a diameter of 0.5-3 mm that does not blanche with pressure and is not palpable</td>
<td>[ ] No</td>
</tr>
<tr>
<td>Petechiae (does not include steroid-induced or senile purpura)</td>
<td>[ ] Less than or equal to 10 in a patient's palm-sized area in the most affected body area [ ] Any number if reported by the patient</td>
<td>[ ] More than 10 in a patient's palm-sized area or more than 5 in at least 2 patient's palm-sized areas located in at least 2 different body areas, one above and one below the belt (in the most affected body areas)</td>
</tr>
<tr>
<td>Ecchymoses (purpuric macule, bruises, or contusions)</td>
<td>Flat, rounded, or irregular red, blue, purplish, or yellowish green patch, larger than a petechia. Elevation indicated spreading of an underlying hematoma into the superficial layers of the skin</td>
<td>[ ] None or up to 2 in the same body area, but smaller than a patient's palm-sized area, if (a) spontaneous or (b) disproportionate to trauma /constriction</td>
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<tr>
<td>Subcutaneous hematomas</td>
<td>Bulging localized accumulation of blood, often with discoloration of overlying skin</td>
<td>[ ] No</td>
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<tr>
<th>Bleeding from minor wounds</th>
<th>[ ] No</th>
<th>[ ] Lasting ≤5 min</th>
<th>[ ] Lasting &gt;5 min or interfering with daily activities</th>
<th>[ ] Requiring protracted medical observation at the time of this visit</th>
<th>[ ] Medical report describing patient's evaluation by a physician</th>
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<tr>
<td></td>
<td></td>
<td>[ ] Any episode if reported by the patient</td>
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| Mucosa | | | | |
|--------|--------|-------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|

<p>| Petechiae, purpuric macules, and ecchymosis | Same as for skin | | | | |</p>
<table>
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<tr>
<th>Epistaxis</th>
<th>Any bleeding from the nose may be anterior or posterior and unilateral or bilateral</th>
<th>[ ] No</th>
<th>[ ] Lasting ≤5 min</th>
<th>[ ] Lasting &gt;5 min or interfering with daily activities</th>
<th>[ ] Packing or cauterization or in-hospital evaluation at the time of this visit</th>
<th>[ ] Medical report describing packing or cauterization or in-hospital evaluation</th>
<th>[ ] RBC transfusion or Hb drop &gt;2 g/dL</th>
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<tr>
<td>Oral cavity, gum bleeding</td>
<td>Any bleeding from the gingival margins</td>
<td>[ ] No</td>
<td>[ ] Lasting ≤5 min</td>
<td>[ ] Lasting &gt;5 min or interfering with daily activities</td>
<td>[ ] Requiring protracted medical observation at the time of this visit</td>
<td>[ ] Medical report describing patient's evaluation by a physician</td>
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<tr>
<td>Oral cavity, hemorrhagic bullae or blisters</td>
<td>Visible raised, thin-walled, circumscribed lesion containing blood. Each bulla (&gt;5 mm) is larger than a vesicle. Bullae, vesicles, and blisters should be counted together as bulla</td>
<td>[ ] No</td>
<td>[ ] Less than 3</td>
<td>[ ] From 3 to 10 but no difficulty with mastication</td>
<td>[ ] More than 10 or more than 5 if difficulty with mastication</td>
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<tr>
<td>Oral cavity, bleeding from bites to lips and tongue or after deciduous tooth loss</td>
<td>Any localized collection of blood visible, palpable, or revealed by imaging. May dissect through fascial planes</td>
<td>[ ] No</td>
<td>[ ] Lasting ≤ 5 min</td>
<td>[ ] Lasting &gt; 5 min or interfering with daily activities</td>
<td>[ ] Interventions to ensure hemostasis or in-hospital evaluation at the time of this visit</td>
<td>[ ] Medical report describing interventions to ensure hemostasis or in-hospital evaluation</td>
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<tr>
<td>Condition</td>
<td>Description</td>
<td>Yes</td>
<td>No</td>
<td>Possibly</td>
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<td><strong>Subconjunctival hemorrhage (not due to conjunctival disease)</strong></td>
<td>Bright red discoloration underneath the conjunctiva at onset; may assume the appearance of an ecchymosis over time</td>
<td>[ ] No</td>
<td>[ ] Yes</td>
<td>[ ] Petechiae/hemorrhage partially involving 1 eye</td>
<td>[ ] Any episode if reported by the patient</td>
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<td>[ ] Petechiae/hemorrhage partially involving both eyes, or diffuse hemorrhage in 1 eye</td>
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<td>[ ] Diffuse hemorrhage in both eyes</td>
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<tr>
<td><strong>Organ (and internal mucosae)</strong></td>
<td>GI bleeding not explained by visible mucosal bleeding or lesion: hematemesis, melena, hematochezia, rectorrhagia</td>
<td>[ ] No</td>
<td>[ ] Yes</td>
<td>[ ] Any episode if reported by the patient</td>
<td>[ ] Present at the visit</td>
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<td>[ ] Requiring endoscopy or other therapeutic procedures or in-hospital evaluation at the time of this visit</td>
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<td>[ ] Medical report prescribing endoscopy or other therapeutic procedures or in-hospital evaluation</td>
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<td>[ ] RBC transfusion or Hb drop &gt;2 g/dL</td>
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<tr>
<td>Condition</td>
<td>[ ] No</td>
<td>[ ] Any episode if reported by the patient</td>
<td>[ ] Present at this visit</td>
<td>[ ] Requiring bronchoscopy or other therapeutic procedures or in-hospital evaluation at the time of this visit</td>
<td>[ ] RBC transfusion or Hb drop &gt;2 g/dL</td>
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<td>Lung bleeding</td>
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<td>Hemoptysis</td>
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<td>Tracheobronchial bleeding</td>
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<tr>
<td>Hematuria</td>
<td>[ ] No</td>
<td>[ ] Any episode if reported by the patient</td>
<td>[ ] Macroscopic (laboratory analysis)</td>
<td>[ ] Macroscopic, and requiring cystoscopy or other therapeutic procedures or in-hospital evaluation at the time of this visit</td>
<td>[ ] RBC transfusion or Hb drop &gt;2 g/dL</td>
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<td>Menorrhagia (compared with pre-ITP or to a phase of disease with normal platelet count)</td>
<td>[ ] No</td>
<td>[ ] Doubling number of pads or tampons in last cycle compared with pre-ITP or to a phase of disease with normal platelet count</td>
<td>[ ] Changing pads more frequently than every 2 h or clot and flooding</td>
<td>[ ] Requiring combined treatment with antifibrinolytics and hormonal therapy or gynecologic investigation (either at this visit or described in a medical report)</td>
<td>[ ] RBC transfusion or Hb drop &gt;2 g/dL</td>
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<tr>
<td>Intramuscular hematomas (only if diagnosed by a physician with an objective method)</td>
<td>[ ] No</td>
<td>[ ] Post trauma, diagnosed at this visit, if judged disproportionate to trauma</td>
<td>[ ] Spontaneous, diagnosed at this visit</td>
<td>[ ] Spontaneous or post trauma (if judged disproportionate to trauma) diagnosed at this visit and requiring hospital admission or surgical intervention, [ ] An equivalent episode if described in a medical report</td>
<td>[ ] RBC transfusion or Hb drop &gt;2 g/dL</td>
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<tr>
<td>Hemarthrosis (only if diagnosed by a physician with an objective method)</td>
<td>[ ] No</td>
<td>[ ] Post trauma, diagnosed at this visit, function conserved or minimally impaired, if judged disproportionate to trauma</td>
<td>[ ] Spontaneous, diagnosed at this visit, function conserved or minimally impaired</td>
<td>[ ] Spontaneous or post trauma (if judged disproportionate to trauma), diagnosed at this visit and requiring immobilization or joint aspiration</td>
<td>[ ] Spontaneous or post trauma (if judged disproportionate to trauma) diagnosed at this visit and requiring surgical intervention</td>
<td>[ ] An equivalent episode if described in a medical report</td>
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<td>Medical Condition</td>
<td>Diagnosis Method</td>
<td>Description</td>
<td>Medical Report</td>
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<td>Ocular bleeding</td>
<td>(only if diagnosed by a physician with an objective method)</td>
<td>[ ] No</td>
<td>[ ] Any post trauma vitreous or retinal hemorrhage involving one or both eyes with or without impaired/blurred vision present at this visit if judged disproportionate to trauma</td>
<td>[ ] An equivalent episode if described in a medical report</td>
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<td>[ ] Spontaneous vitreous or retinal hemorrhage involving one or both eyes with impaired/blurred vision present at this visit</td>
<td>[ ] An equivalent episode if described in a medical report</td>
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<td>[ ] Spontaneous vitreous or retinal hemorrhage with loss of vision in one or both eyes present at this visit</td>
<td>[ ] An equivalent episode if described in a medical report</td>
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<tr>
<td>Intracranial bleeding</td>
<td></td>
<td>[ ] No</td>
<td>[ ] Any post trauma event requiring hospitalization</td>
<td>[ ] Any spontaneous event requiring hospitalization in the presence of an underlying intracranial lesion</td>
<td>[ ] Any spontaneous event requiring hospitalization without an underlying intracranial lesion</td>
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<td>Other internal bleeding: hemoperitoneum, hemopericardium, hemothorax, retroperitoneal bleeding, hepatic and splenic peliosis with organ rupture, retro-orbital bleeding metrorrhagia etc.</td>
<td></td>
<td></td>
<td>[ ] Any event requiring hospitalization &lt;48 h</td>
<td>[ ] Any event requiring hospitalization &gt;48 h or RBC transfusion or Hb drop &gt;2 g/dL</td>
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