IMPROVED MOUSE MODELS FOR THE STUDY OF TREATMENT MODALITIES USING SULFUR-CONTAINING SMALL-MOLECULAR-WEIGHT MOLECULES FOR PASSIVE IMMUNE-MEDIATED THROMBOCYTOPENIA

by

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Improved Mouse Models for the Study of Treatment Modalities using Sulfur-Containing Small-Molecular-Weight Molecules for Passive Immune-Mediated Thrombocytopenia

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Abstract

Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by autoantibody-mediated platelet destruction. To test the efficacy of novel sulfur compounds as alternative treatments for ITP, we used a mouse model of passive immune thrombocytopenia (PIT). Using this model, the platelet nadir could not be maintained, with platelet counts rising after day 4, despite daily anti-platelet antibody administration. We examined reticulated platelet counts by flow cytometry, and found increased thrombopoiesis in the bone marrow to be at least partially responsible for this platelet rebound. Consequentially, two improved mouse models of PIT were developed, where the platelet rebound is circumvented. The first model employs sub-lethal total body γ-irradiation in combination with daily antibody administration, while the second model employs gradual escalation of the daily antibody dose. Finally, we show that none of the tested candidate compounds show efficacy in elevating platelet counts *in vivo*, likely due to their limited solubility.

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Table of Contents

Section **Page Number**

The University of Toronto Authority to Distribute Form …….

Title Page……..i

Abstract……...…ii

Acknowledgements……iii

Table of Contents……..……..…iv

List of Tables…….....…viii

List of Figures……...……ix

List of Appendices……..x

List of Abbreviations……..xi

Chapter 1 Introduction…….……...…...1

1.1 Overview……...1

1.2 Immune cytopenias……....3

1.3 Mononuclear Phagocyte System (MPS)……...…....4

1.4 Human and Mouse Fcγ Receptors (FcγRs)……...……6

1.5 Immune Thrombocytopenic Purpura (ITP)……...……..10

1.5.1 Pathophysiology of ITP…….10

1.5.2 Classification and underlying causes of ITP……....…13

1.5.3 Available treatments……..……14

1.6 Intravenous immunoglobulins, IVIG and anti-D……..……..17

1.6.1 Preparation of IVIG and anti-D……...……..17

1.6.2 Mechanism of Action of IVIG……..…18

1.6.3 Mechanism of action of anti-D……..24

1.6.4 Disadvantages of Intravenous Immunoglobulins……...25

1.6.5 Alternatives to Intravenous Immunoglobulins……..…….26

1.7 Small-Molecular-Weight Compounds as potential alternatives to immunoglobulin therapies……...……27

1.8 Mouse models of ITP……..30
1.9 Hypotheses…….……32
1.10 Specific Objectives…….…….33

Chapter 2 Materials and Methods……....34
2.1 Mice and Husbandry……...……34
2.2 Murine models of passive-immune thrombocytopenia (PIT)…….....35
2.2.1 Published model of PIT…….35
2.2.2 Total Body γ-irradiation combination model……..…….....35
2.2.3 Dose escalation model of PIT……..……..35
2.3 Blood collection…….…….36
2.3.1 Method 1……...……...36
2.3.2 Method 2……...36
2.4 Data collection and analysis……..……..37
2.4.1 Platelet enumeration……...……...37
2.4.2 Enumeration of Reticulated Platelets……...…….40
2.4.3 Data and Statistical Analysis……...……..43
2.5 Treatments…….…….44
2.5.1 IVIG……..44
2.5.2 Anti-TER-119……44
2.5.3 Small-Molecular-Weight Sulfur Compounds……...…….……45
2.5.3.1 Maximum Tolerated Doses (MTDs)……..48
2.5.3.2 Compound Preparation, Solubility and Administration…….49

Chapter 3 Results…….53
3.1 Method of Blood collection…….……53
3.2 The dose of anti-CD41 administered must be adjusted by mouse weight……..56
3.3 Spontaneous platelet recovery is a result of increased thrombopoiesis in the bone marrow…….……58
3.3.1 Immune response to anti-CD41 antibody is not the underlying cause of platelet rebound……...…58

3.3.2 Spontaneous platelet recovery correlates with increased reticulated platelets counts in CD1 and Balb/c mice……...60
3.3.3 There is an abundance of available epitopes for anti-CD41 binding, with only 30% of platelets being bound by the anti-CD41 antibody at any given time……...…….63
3.4 New mouse model of ITP #1: Sub-lethal total body irradiation in combination with passive daily anti-platelet antibody administration……65
3.4.1 TBI combination model effectively prevents the platelet rebound, maintaining consistently low levels of thrombocytopenia over the course of the experiment……65
3.4.2 Daily administration of anti-CD41 antibody is necessary for continuous maintenance of antibody-induced thrombocytopenia…….....69
3.4.3 The TBI mouse model fails to respond to IVIG but responds to anti-TER-119 treatment…….……71
3.5 New mouse model of ITP #2: Gradual escalation of the anti-platelet antibody dose over the course of the experiment……74
3.5.1 Dose escalation of anti-CD41 antibody effectively prevents the platelet rebound…….……..74
3.5.2 IVIG is effective at increasing platelet counts using the dose-escalation mouse model……78
3.5.3 Higher doses of anti-platelet antibody may adversely affect magakaryocytes in the bone marrow……...……..81
3.6 The efficacy of small-molecular-weight compounds *in vivo*……..83
3.6.1 Thimerosal has no efficacy in elevating platelet counts in both the TBI-combination and the dose escalation models of PIT……83
3.6.2 Efficacy of first generation compounds using the dose escalation model of PIT……..87
3.6.3 Efficacy of second generation compounds using the dose escalation model of PIT…….……91
3.6.4 Toxicity of tested small molecular weight compounds……...…..94

Chapter 4 Discussion……..…..…..95
4.1 Spontaneous platelet recovery is a result of increased thrombopoiesis……....95
4.2 Sub-lethal total body irradiation in combination with passive daily anti-platelet antibody administration is effective at preventing a spontaneous platelet rebound……..…....100
4.3 Gradual dose escalation of the anti-platelet antibody successfully maintains the antibody-induced thrombocytopenia..……..……..……..104
4.4 Efficacy of small-molecular-weight compounds *in vivo*……..…..….106
Chapter 5 Summary and Future Directions……..……..…..110
Chapter 6 References……...……....113
Appendix I Structures of Additional Newly-Synthesized Chemical Compounds……..…132
Appendix II Response of individual mice to the tested small molecular-weight compounds under various conditions (by experiment)……..…….134
Appendix III IVIG administration on day 0, delays the anti-CD41 induced drop in platelet counts……..……143
Appendix IV Animal Use Protocol Approval……..…….144

List of Tables

Table **Page Number**

Table 1.4 Expression of Human and Mouse FcγRs on various Cell Populations…….9

Table 2.5.3.2 Candidate chemicals: Preparation, Solubility and mode of Administration…...51

Table 3.1 Comparison of two methods of blood collection and FACS analysis……....54

List of Figures

Figure **Page Number**

Figure 1.6.2 Current paradigm of a possible IVIG mechanism of action……..….22

Figure 2.4.1 FACS Acquisition and Analysis Template for Platelet Enumeration……38

Figure 2.4.2 Template Setup for Reticulated Platelet Enumeration……...41

Figure 2.5.3 Chemical Structures of Tested Small-Molecular-Weight Compounds……..46

Figure 3.1 Comparison of Two Methods of Blood Collection……..…55

Figure 3.2 The dose of anti-CD41 must be adjusted by mouse weight……...57

Figure 3.3.1 Effect of daily anti-platelet antibody administration on platelet counts in CB.17 SCID mice……...59

Figure 3.3.2 Effect of daily anti-platelet antibody administration on total and reticulated platelet counts in CD1 and Balb/c mice……..…..61

Figure 3.3.3 The proportion of platelets coated with rat anti-CD41 antibody does not change over time……...64

Figure 3.4.1 Sub-lethal TBI mouse model of PIT……..….67

Figure 3.4.2 Necessity of daily anti-platelet antibody administration…….……70

Figure 3.4.3 Anti-TER-119 treatment is effective but IVIG is ineffective when using the TBI-combination model …….……....72

Figure 3.5.1 Comparison of anti-CD41 dose-escalation to same-dose mouse model of PIT……...……76

Figure 3.5.2 IVIG treatment shows efficacy in the anti-CD41 dose-escalation mouse model……..…79

Figure 3.5.3 IVIG efficacy using a modified dose-escalation protocol…….…….82

Figure 3.6.1 Thimerosal efficacy using the TBI-combination and the dose escalation models of PIT…….....85

Figure 3.6.2 Efficacy of first-generation small-molecular-weight compounds using the dose-escalation model of PIT……..89

Figure 3.6.3 Efficacy of second-generation small-molecular-weight compounds using the dose-escalation model of PIT……..92

List of Appendices

Appendix **Page Number**

Appendix I Structures of Additional Newly-Synthesized Chemical Compounds……..…132

Appendix II Response of individual mice to the tested small molecular-weight sulfur compounds under various conditions (by experiment)……...…….134

Appendix II.1 Efficacy of first-generation small-molecular-weight compounds when administered IV to Balb/c mice…….135

Appendix II.2 Efficacy of first-generation small-molecular-weight compounds when administered IP to Balb/c mice……..137

Appendix II.3 Efficacy of 2,4-dinitrophenyl methyl disulfide (C7) when administered IP to Balb/c mice……139

Appendix II.4 Efficacy of second generation sodium salts when administered IP to Balb/c mice……...141

Appendix III IVIG administration on day 0, delays the anti-CD41 induced drop in platelet counts……..……143

Appendix IV Animal Use Protocol Approval……..……144

List of Abbreviations

ADCC	Antibody-dependent cell cytotoxicity
AIHA	Autoimmune hemolytic anemia
AIN	Autoimmune neutrophenia
CPDA	Citrate-phosphate-dextrose-adenine
CR	Complement receptor
CSF	Colony-stimulating factor
CSF-1	Macrophage colony-stimulating factor
DCs	Dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
FACS	Fluorescence activated cell sorting
FcγR	Fc- gamma receptor
FcRn	Neonatal Fc receptor
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	Glycoprotein
GPI	Glycosyl-phosphatidyl-inositol
Gy	Gray
HDN	Hemolytic disease of the newborn
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneal
ITAM	Immuno-receptor tyrosine-based activation motif
ITIM	Immuno-receptor tyrosine-based inhibition motif
ITP	Immune thrombocytopenic purpura
IV	Intravenous

IVIG	Intravenous immunoglobulin
Mφ	Monocyte-macrophage
MCP-1	Monocyte-chemoattractant protein-1
MHC	Major histocompatibility complex
MMA	Monocyte monolayer assay
MPS	Mononuclear phagocyte system
MTD	Maximum tolerated dose
NK	Natural Killer (cells)
PBS	Phosphate-buffered saline
PGE_2	Prostaglandin E2
PIT	Passive immune thrombocytopenia
RBCs	Red blood cells
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
SH2	Src homology 2
SHIP	Src homology domain 2-containing inositol-5-phosphatase
SHP-1	Src homology domain 2-containing protein tyrosine phosphatase 1
SIGN	Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
SIGN-R1	SIGN homolog (SIGN related-1)
sICs	Soluble immune complexes
SSC	Side scatter
TBI	Total body γ-irradiation
Th1/Th2	T helper cells
TNFα	Tumor necrosis factor-alpha
TO	Thiazole orange
TPO	Thrombopoietin

Chapter 1

Introduction

1.1 Overview

Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by Fcγ-receptor (FcγR)-mediated clearance of autoantibody sensitized platelets (McMillan, R., 2000b, Cines, D.B. & Blanchette, V.S., 2002). Available treatments for ITP management include splenectomy, corticosteroids, immunoglobulins (IVIG or anti-D), cytotoxic drugs, anti-CD20 and thrombopoietin receptor agonists (Chong, B.H. & Ho, S.J., 2005, Stasi, R. *et al.*, 2008). However, these treatments all have associated disadvantages, including a range of side effects and limited efficacy. Although immunoglobulin therapies (IVIG and anti-D) have become a common treatment for ITP as well as for a range of other autoimmune diseases (Negi, V.S. *et al.*, 2007), their mechanism of action is still unknown (Nimmerjahn, F. & Ravetch, J.V., 2008). In addition, immunoglobulin therapy use is associated with high cost, worldwide shortages due to their acquisition from human donations, and with a rare but not trivial potential for infectious disease transmission. Thus, much of the current research is focused on discerning the mechanisms of action of immunoglobulin therapies, and on development of improved therapeutic approaches for ITP. The aim of this thesis is to test the efficacy of novel small-molecular weight compounds, which are non-human-derived and less expensive to produce, as potential alternatives to existing immunoglobulin therapies.

Earlier work in our laboratory indicated that sulfur-containing small-molecular-weight molecules that target sulfur-containing amino acids on the surface of mononuclear phagocytes can effectively inhibit the phagocytosis of antibody-coated red cells *in vitro* by inhibiting binding of the antibody Fc to the monocyte-macrophage (Mφ) FcγRs (Rampersad, G.C. *et al.*, 2005). The aim of this project was to test the efficacy of newly synthesized (SH) and disulfide (SS) compounds using a widely employed mouse model of passive immune thrombocytopenia (PIT) (Crow, A.R. *et al.*, 2001).

In this model, immune-mediated platelet destruction is induced by passive daily administration of an anti-platelet antibody, prior to treatment administration for reversal of platelet destruction (Crow, A.R. *et al.*, 2001). Unfortunately, we found that using this model, the antibody-induced thrombocytopenia could not be maintained after day 4 of the experiment, with platelet counts 'spontaneously' rising in face of daily anti-platelet antibody administration (Foo, A.H., 2006). This precluded accurate interpretation of results for evaluation of various treatment modalities. As we needed a sensitive and consistent model that will allow us to discern subtle drug efficacy, we first had to identify the underlying cause of platelet rebound in this mouse model. Thus, one of the aims of this thesis was to develop an improved mouse model of PIT for *in vivo* studies where antibody-induced thrombocytopenia can consistently be maintained close to the nadir over a prolonged period of time.

1.2 Immune cytopenias

Immune cytopenias are conditions that involve clearance of specific hematopoietic cells within the blood by auto- or allo-antibodies (Gilliland, B.C. & Evans, R.S., 1973, Trivedi, D.H. & Bussel, J.B., 2003). The B lymphocytes in the patients' blood produce IgG antibodies that coat (opsonize) specific blood cells, and target them for recognition by the Fcγ receptors (FcγRs) on monocyte-macrophage (Mφ) membranes (Gilliland, B.C. & Evans, R.S., 1973, Aster, R.H., 2005). This results in phagocytosis and subsequent intracellular destruction of these cells by the mononuclear phagocyte system (MPS) (Petz, L.D. & Garratty, G., 2004). Antibody-mediated cell destruction may also occur via Fc-independent pathways, including Fc-independent phagocytosis (Webster, M.L., 2008), complement activation (Hed, J., 1998), or cell-mediated cytotoxicity (Olsson, B. *et al.*, 2003).

Examples of immune cytopenias include immune thrombocytopenic purpura (ITP, where platelets are destroyed), autoimmune hemolytic anemia (AIHA, where red blood cells are destroyed), and autoimmune neutrophenia (AIN, where neutrophils are destroyed). Immune cytopenias also include alloimmune hemolytic anemias resulting from transfusion reactions, where patients produce alloantibodies to the donor red cell antigens, and hemolytic disease of the newborn (HDN) that results from maternal antibodies crossing the placenta (Marsh, J.C. & Gordon-Smith, E.C., 2001). The extra-vascular destruction of the antibody-opsonized blood cells by the MPS is the hallmark of immune cytopenias and it can be rapid and life-threatening.

1.3 Mononuclear Phagocyte System (MPS)

Cells that comprise the MPS are classified based on similar morphology, enzyme expression profiles, presence of receptors for the Fc domain of immunoglobulins, and non-specific particle uptake ability through endocytosis (Hume, D.A., 2006). MPS includes macrophages, monocytes, their lineage-committed precursors (Hume, D.A., 2006), and in some definitions may include MPS derivatives, such as osteoclasts, dendritic cells (DCs), and Langerhans cells (Aqel, N.M. *et al.*, 1987).

The hematopoietic process that gives rise to macrophages, DCs and osteoclasts, starts with hematopoietic stem cells within the adult bone marrow. Initially, granulocyte/ macrophage colony forming units differentiate into monoblasts that later become pro-monocytes, and eventually give rise to mature monocytes that are released into the circulation (Gordon, S. & Taylor, P.R., 2005). Monocytes within the circulation traffic to specific tissues, and enter by transendothelial migration (regulated by chemokines that activate a range of leukocyte integrins and by leukocyte-adhesion molecules) (Imhof, B.A. & Aurrand-Lions, M., 2004). Monocytes differentiate into macrophages in resident tissues, including the lung (alveolar macrophages), liver (Kupffer cells), brain (microglia), testis, kidney, pancreas, and spleen (Hume, D.A. *et al.*, 2002). This differentiation is directed by colony-stimulating factors (CSFs), including macrophage CSF (CSF-1) and granulocyte-macrophage CSF (GM-CSF) (Hume, D.A., 2008).

Macrophages play an integral role in immune responses, inflammation and tissue homeostasis (Hume, D.A. *et al.*, 2002). As part of the innate immune response they are able to secrete pro- and anti-inflammatory cytokines (such as interleukin (IL)-1, IL-6 or IL-10, respectively) to regulate the differentiation and function of surrounding cells (Hume, D.A., 2006, Gordon, S. &

Taylor, P.R., 2005). In addition, macrophages are responsible for phagocytosis of pathogens and dead cells resulting from infection, wounding, or normal cell turnover (Park, J.B., 2003). Phagocytosis of pathogens often results in antigen presentation and stimulation of the adaptive immune response (Hume, D.A. *et al.*, 2002). Clearance of antibody-coated cells by mononuclear phagocytes is the underlying cause of immune cytopenias (Cines, D.B. & Blanchette, V.S., 2002). Macrophages express complement receptors (CR1, CR3 and CR4) which are instrumental in recognition and phagocytosis of complement (C3b or C3bi) coated cells (Aderem, A. & Underhill, D.M., 1999). In addition, clearance of IgG antibody-coated cells is primarily mediated through FcγRs on macrophage surfaces (Park, J.B., 2003, Aderem, A. & Underhill, D.M., 1999), predominantly within the spleen (Hume, D.A., 2006). However, Fc-independent phagocytosis has also been implicated in the clearance of antibody-coated cells (Webster, M.L., 2008).

1.4 Human and Mouse Fcγ Receptors (FcγRs)

FcγRs are surface glycoproteins that belong to the Ig super-family and are predominantly expressed on leucocytes (Siberil, S. *et al.*, 2007). They are important immune regulators that play a central role in phagocytosis of antibody-coated cells or pathogens (Park, J.B., 2003, Aderem, A. & Underhill, D.M., 1999, Schmidt, R.E. & Gessner, J.E., 2005). FcγRs can be broadly subdivided into two categories based on their structure and function, as activating or inhibitory receptors. The activating FcγRs contain an immuno-receptor tyrosine-based activation motif (ITAM) in their cytoplasmic tails or in the cytoplasmic domain of the γ-chain. Phosphorylation of this motif by Src-family protein tyrosine kinases (e.g. Lyn or Fyn), results in its association with Src homology 2 (SH2) domain-containing cytosolic tyrosine kinases of the Syk family, which in turn lead to cell activation (Cambier, J.C., 1995). In contrast, the inhibitory FcγRs contain an immuno-receptor tyrosine-based inhibition motif (ITIM), phosphorylation of which results in the recruitment of SH2-domain containing phosphatases, including SHP-1 and SHIP (Vivier, E. & Daeron, M., 1997). These phosphatases act to down-regulate activating tyrosine phosphorylation. Activating and inhibitory receptors are usually co-expressed on the cell surface, and function in concert with one another (Daeron, M. *et al.*, 2008).

Human activating FcγRs, which include FcγRI, FcγRIIA, FcγRIIC, and FcγRIII, play an integral role in the initiation of phagocytosis (Young, J.D. *et al.*, 1984), which in turn may lead to antigen presentation (Hamano, Y. *et al.*, 2000). The inhibitory FcγRIIB down-regulates activation responses, terminates IgG-mediated effector cell responses, and maintains peripheral tolerance (Ravetch, J.V. & Bolland, S., 2001). Different hematopoietic cells express different classes and isotypes of FcγRs as summarized in Table 1.4. All FcγRs can recognize the Fc portion of IgG, and bind strongly to IgG3 and IgG1, while exhibiting a low affinity for IgG2. In comparison,

IgG4 binds mostly to FcγRI and FcγRIIB, with an intermediate and weak affinity respectively (Siberil, S. *et al.*, 2007).

FcγRI (also known as CD64) is a 72 kDa high affinity receptor that binds both monomeric IgG and immune complexes (which are usually comprised of IgG antibodies and their corresponding antigens). FcγRI transduces activation signals leading to antibody-dependent cell-mediated cytotoxicity (ADCC), endocytosis, and phagocytosis (Peltz, G. *et al.*, 1988).

FcγRIII (also known as CD16) is a 40-43 kDa receptor, which can be found in two isoforms with an intermediate affinity for IgG. FcγRIIIA is a transmembrane isoform that similarly to FcγRI, is involved in ADCC, phagocytosis, endocytosis and cytokine release (Siberil, S. *et al.*, 2007). In comparison, the glycosyl-phosphatidylinositol (GPI) anchored FcγRIIIB isoform is involved in de-granulation and generation of reactive oxygen intermediates (Salmon, J.E. *et al.*, 1995).

FcγRII (also known as CD32) has a low affinity for IgG, and is the most abundant FcγR on hematopoietic cells. It has two activating (FcγRIIA and FcγRIIC), and two inhibitory (FcγRIIB1 and FcγRIIB2) isoforms. FcγRIIA is implicated in endocytosis initiation, phagocytosis, ADCC, and inflammatory mediator release, while the inhibitory FcγRIIB1 and FcγRIIB2 co-ligate with activating receptors, transducing inhibitory signals that down-regulate immune functions (including B-cell and mast-cell activation) (Siberil, S. *et al.*, 2007).

Mice express homologs of some human FcγRs on their mononuclear phagocytes (Gessner, J.E. *et al.*, 1998), including FcγRI, FcγRIIB (FcγRII in mice), and FcγRIIIA (FcγRIII in mice). In addition, mice express an activating FcγRIV, which is absent in humans (Nimmerjahn, F. *et al.*, 2005). Similarly to humans, mouse FcγRI and FcγRIII are activating receptors that bind IgG with

a high and low affinity, respectively, whereas FcγRII is an inhibitory receptor that down-regulates phagocytosis (Amigorena, S. *et al.*, 1992), and has a low affinity for IgG binding.

All mouse FcγRs can bind to mouse IgG1, IgG2a, and IgG2b, as well as to human IgG1 and IgG3. IgG2a has a high affinity for FcγRI, whereas IgG1 and IgG2b preferentially bind to FcγRII and FcγRIII (Teillaud, J.L. *et al.*, 1985). Mouse IgG3 binds to FcγRI, but not to FcγRII or FcγRIII (Gavin, A.L. *et al*, 1998). FcγRIV binds with an intermediate affinity to mouse IgG2a and IgG2b (Nimmerjahn, F. *et al.*, 2005). Generally, IgG2a and IgG2b interact with FcγRs to mediate responses such as clearance of antibody-coated particles (Uchida, J. *et al.*, 2004).

Table 1.4 Expression of Human and Mouse FcγRs on various Cell Populations

	hFcγRI	hFcγRII	hFcγRIII	mFcγRI	mFcγRII	mFcγRIII	mFcγRIV
Myeloid Progenitors	✔						✔
Monocytes	✔	FcγRIIA FcγRIIB	FcγRIIIA				✔
Macrophages	✔	FcγRIIA FcγRIIB	FcγRIIIA	✔	✔	✔	✔
Dendritic cells (DCs)	✔	FcγRIIB					✔
B cells		FcγRIIB			✔		
Subpopulation of T cells			FcγRIIIA		✔	✔	
Mast cells		FcγRIIB			✔	✔	
Basophils		FcγRIIB			✔		
Eosinophils					✔	✔	
Neutrophils		FcγRIIA	FcγRIIIB			✔	
Platelets		FcγRIIA					
Natural Killer (NK) cells		FcγRIIC	FcγRIIIA			✔	
Mesangial cells				✔	✔	✔	

Table 1.4 Expression of human (indicated by 'h') and mouse (indicated by 'm') FcγR classes on various hematopoietic and related cells. For some FcγR classes, specific isotype expression is indicated. This information was compiled from (Gessner, J.E. *et al.*, 1998) and (Siberil, S. *et al.*, 2007).

1.5 Immune Thrombocytopenic Purpura (ITP)

Immune thrombocytopenic purpura (ITP) is an autoimmune cytopenia in which autoantibody-mediated platelet clearance occurs predominantly via FcγR-mediated phagocytosis, resulting in platelet destruction primarily within the spleen (Cines, D.B. & Blanchette, V.S., 2002, McMillan, R., 2000a). In addition, platelets may also be cleared by natural killer (NK) cells, monocytes or eosinophils via ADCC (Garcia-Suarez, J. *et al.*, 1993), by T-cell mediated cytotoxicity (Olsson, B. *et al.*, 2003, Olsson, B. *et al.*, 2008) and/or in an Fc-independent fashion (Nieswandt, B. *et al.*, 2000),. This disease affects approximately 1 in 10,000 people per year, with about half of these cases occurring in children (Frederiksen, H. & Schmidt, K., 1999).

1.5.1 Pathophysiology of ITP

Platelets have an important role in blood homeostasis and vascular repair. Clinical symptoms of ITP usually appear when platelet counts drop from normal levels of 150–450 x 10^9/L to below 50 x 10^9/L (Cines, D.B. & McMillan, R., 2005). As ITP is a heterogeneous disease, the clinical symptoms vary in duration and severity between patients, and include purpura, petechiae, ecchymoses, mucosal bleeding, and potentially life-threatening complications due to intracranial hemorrhage (Cines, D.B. & Blanchette, V.S., 2002).

The first evidence that ITP was caused by a plasma-derived antiplatelet factor was provided by Harrington, W.J. *et al.* (1951), who showed that infusion of plasma from patients with ITP, induced thrombocytopaenia in normal recipients. Research over the years have shown that the pathology of ITP is primarily due to accelerated clearance of auto-antibody coated platelets via FcγR-mediated phagocytosis, predominantly within the spleen and liver (Cooper, N. & Bussel, J., 2006). Anti-platelet antibodies (mainly IgG, and occasionally IgM and IgA) recognize one or

more glycoprotein (GP) antigens on the platelet surface. Most ITP patients have auto-antibodies targeted to GPIIb/IIIa and/or to GPIb/IX, with some patients also exhibiting antibodies targeted to GPIV and/or GPIa/IIa (He, R. *et al.*, 1994). Digested membrane peptides (GPIIb/IIIa and GP Ib-IX) of phagocytosed platelets are presented by macrophages to specific clones of CD4+ T cells. These T cells become activated and exert helper activity on auto-reactive antibody-producing B cells, causing them to produce more auto-antibodies directed against platelets (Semple, J.W. & Freedman, J., 1991, Kuwana, M. *et al.*, 2001). T cell activation plays an important role in this phagocytic cycle, as it leads to an increased CD154 expression, which in turn binds to CD40 on B cells. This interaction is essential for B cell proliferation, isotype switching, memory cell generation and autoantibody production (Imbach, P. & Kuhne, T., 1998, Neron, S. *et al.*, 2006).

Also, adults with ITP often have increased numbers of HLA-DR+ T cells, which may stimulate synthesis of anti-HLA antibodies following exposure to GPIIb/IIIa fragments (Cines, D.B. & Blanchette, V.S., 2002). As HLA-DR antigens are expressed on platelets and microparticles in ITP patients, the anti-HLA antibodies likely contribute to platelet destruction (Coopamah, M.D. *et al.*, 2003). This is supported by the finding that the percentage of HLA-DR+ platelets is inversely proportional to the platelet count (Semple, J.W. *et al.*, 1996).

In addition, recent reports have suggested that Fc-independent phagocytosis may play a significant role in ITP pathogenesis, as antibodies directed against platelet GPIbα were shown to induce thrombocytopenia in an Fc-independent manner (Nieswandt, B. *et al.*, 2000). One alternative mechanism of antibody-mediated platelet clearance is through T-cell cytotoxicity. Olsson, B. *et al.* have demonstrated that T lymphocytes from ITP patients can induce platelet lysis (2003), and that ITP patients have increased numbers of $CD3^+$ T cells, and increased surface expression of factors involved in T-cell homing (VLA-4 and CX3Cr1) (2008). Another

mechanism of antibody-mediated platelet clearance is through ADCC, which results in lysis of antibody-coated platelets by activated NK cells or monocytes (Garcia-Suarez, J. *et al.*, 1993).

In addition to antibody-mediated platelet destruction, platelet production is also likely inhibited by platelet auto-antibodies. Early studies have indicated that platelet turnover, and therefore platelet production, was either decreased or normal in most ITP patients (Heyns Adu, P. *et al.*, 1986, Stoll, D. *et al.*, 1985). As megakaryocytes share the same surface GPs as platelets (GPIIb–IIIa and GPIb–IX) (Vainchenker, W. *et al.*, 1982), adverse effects on megakaryocyte maturation and/or platelet release can be attributed directly to the anti-platelet antibodies in the circulation. *In vitro* studies support this by showing that megakaryocyte growth and maturation could be inhibited by plasma and by purified IgG from patients with chronic ITP (McMillan, R. *et al.*, 2004, Chang, M. *et al.*, 2003). However, it is important to note that the megakaryocytes are not being destroyed in ITP patients, but instead they are being damaged and their function is being impaired.

1.5.2 Classification and underlying causes of ITP

ITP may be classified as primary (idiopathic), or as secondary to an underlying disorder. It may also be classified as acute (six months or less in duration) or chronic (longer than six months). Childhood-onset ITP affects previously healthy young children (with peak age of approximately five years), who usually present with a sudden onset of petechiae or purpura following an infectious illness. Males and females are equally affected, and in more than 70 percent of cases the illness resolves within six months, irrespective of therapy administration or lack thereof. However, adult-onset ITP is chronic in most cases, with an insidious onset. Unlike in children, adult ITP affects approximately twice as many women as men. (Cines, D.B. & Blanchette, V.S., 2002).

Despite significant advances in understanding of ITP, the process that initiates the auto-immune response is still unknown. It may initially be caused by a T-cell disorder, a B-cell abnormality, a thrombopoiesis abnormality, or increased mononuclear phagocyte activation (Cooper, N. & Bussel, J., 2006).

One factor contributing to ITP could be the escape of self-reactive T and B cells through the elaborate control mechanisms instituted by the immune system, to maintain self-tolerance (including central thymic and peripheral tolerance) (Sakaguchi, S. *et al.*, 1995). As a result, these self-reactive B and T cells remain quiescent in the circulation until tolerance is overcome. ITP that develops following an infection is likely a result of molecular mimicry and cross-reactivity, where an antigen on an infectious agent may have a molecular structure similar to a platelet antigen and thereby induce the production of a cross-reacting antibody (Zhou, B. *et al.*, 2005). Alternatively, the infection process may degrade platelet glycoproteins (such as GPIIb/IIIa) to expose cryptic epitopes that initiate the immune response and antibody production. Idiopathic

ITP could be initiated by interruption of checkpoints responsible for removal of auto-reactive antibody producing B cells from the circulation (Wardemann, H. *et al.*, 2003), or, more likely, following the breakdown of suppressor cell and anti-idiotype regulatory networks (Cooper, N. & Bussel, J., 2006).

T cell abnormalities might be major contributors to ITP pathogenesis. First, the observation that the ratios of type1 to type 2 CD4+ helper T cells (Th1/Th2) are increased in patients with ITP, suggested the possibility of ongoing immune activation as part of autoimmunity (Semple, J.W. *et al.*, 1996, Panitsas, F.P. *et al.*, 2004). In addition, cytokine release by T-cells might interfere with megakaryocyte maturation and/or platelet release (Andersson, P.O. *et al.*, 2002). Finally, T cells may exert direct cytotoxic effects on platelets (Olsson, B. *et al.*, 2003) and possibly on megakaryocytes (Olsson, B. *et al.*, 2008), however this mechanism of platelet destruction is, thus far, supported solely by *ex vivo* studies.

1.5.3 Available treatments

Due to the heterogeneity of ITP, there are a range of available therapeutic approaches, which target different aspects of the disease pathogenesis. These treatments include splenectomy, corticosteroids, immunoglobulins (IVIG or anti-D), cytotoxic drugs, anti-CD20 (Rituximab) and thrombopoietin (TPO) receptor agonists. These treatments all have associated disadvantages, including a range of side effects and limited efficacy. The first line of treatment usually involves the administration of corticosteroids (e.g., prednisone, prednisolone, dexamethasone, methylprednisolone) or immunoglobulin preparations (IVIG or anti-D). There is no difference in response rates, response duration or the requirement for splenectomy after 1 year, when any of these treatments are used (George, J.N. *et al.*, 2003, Jacobs, P. *et al.*, 1994).

Corticosteroids work by suppressing cell-mediated and humoral immunity, and down-regulate Fc-mediated platelet clearance through their anti-inflammatory action (George, J.N. *et al.*, 1996, British Committee for Standards in Haematology General Haematology Task Force, 2003). However, side effects associated with prolonged steroid treatment include osteoporosis, impaired glucose tolerance, opportunistic infections and emotional liability. Moreover, their effects are usually short lived as they tend to suppress, but not eliminate, the pathogenic auto-reactive B or T cell clones.

IVIG and anti-D work by different, although not completely understood mechanisms (Cooper, N. *et al.*, 2004a). Accumulating evidence suggests the involvement of IVIG in the blockade of Fc-receptor mediated platelet clearance (Salama, A. *et al.*, 1983, Clarkson, S.B. *et al.*, 1986, Samuelsson, A. *et al.*, 2001), introduction of anti-idiotype antibodies into the circulation (Berchtold, P. *et al.*, 1989), and most recently, up-regulation of the inhibitory FcγRIIB on effector macrophages (Samuelsson, A. *et al.*, 2001) and DC activation (Siragam, V. *et al.*, 2006). Anti-D on the other hand is thought to interfere with platelet phagocytosis via competitive inhibition of Fc receptors on mononuclear phagocytes (Salama, A. *et al.*, 1984, Bussel, J.B. *et al.*, 1991, Ambriz-Fernandez, R. *et al.*, 2002). For a detailed discussion of the mechanisms of action and the disadvantages associated with intravenous immunoglobulins, please refer to chapter 1.6.

A surgical approach to ITP treatment, involves the removal of a major site for platelet sequestration and antibody production - the spleen (Kojouri, K. *et al.*, 2004, Kuwana, M. *et al.*, 2002). Splenectomy results in a long term response rate of 66% (Kojouri, K. *et al.*, 2004), but carries a risk for surgery-associated complications such as surgical mortality, thromboembolic events, and life-threatening sepsis (Dolan, J.P. *et al.*, 2008).

When first-line therapies fail to elevate and maintain platelet counts at a safe level, second-line therapies are used. These include danazol, immunosuppressive agents (azathioprin, mycophenalate mofetil, vinca alkaloids, cyclosporine A, dapsone) and cytotoxic chemotherapy (cyclophosphamide). Some of these therapeutics have transient efficacy, and most, have severe associated side effects (Cines, D.B. & Bussel, J.B., 2005).

In addition, novel therapeutic approaches are being developed and tested, including humanized antibodies (Rituximab and anti-CD40 antibodies) and thrombopoietin (TPO) receptor agonists (Romiplostim and eltrombopag). Rituximab is a chimeric anti-CD20 monoclonal antibody, which induces transient depletion of $CD20^{+}$ B cells and inhibits cellular immunity (Stasi, R. *et al.*, 2007, Cooper, N. *et al.*, 2004). This therapeutic approach is promising, as patients show durable responses, with manageable infusion-related side effects (Arnold, D.M. *et al.*, 2007). Unlike Rituximab, anti-CD40 humanized monoclonal antibodies are targeted to block T-cell and platelet based stimulation of auto-reactive B-cells (Kuwana, M. *et al.*, 2003, Patel, V.L. *et al.*, 2008). The compounds, romiplostim (AMG-531) and eltrombopag (SB-497115), are TPO mimetic agents that stimulate megakaryocytopoiesis and platelet production (Bussel, J.B. *et al.*, 2007, Bussel, J.B. *et al.*, 2009). Romiplostim is a peptibody, which consists of two covalently linked carrier-Fc domains attached to a peptide containing many TPO receptor (c-MPL) - activating sequences, while eltrombopag is a synthetic TPO-receptor agonist. Both TPO mimetic agents have been approved by the US Food and Drug Administration (FDA) in 2008, as they have shown to be efficacious in the treatment of ITP in clinical trials, with mild to moderate side effects (Nurden, A.T. *et al.*, 2009).

1.6 Intravenous immunoglobulins, IVIG and anti-D

1.6.1 Preparation of IVIG and anti-D

Intravenous immunoglobulin (IVIG) is prepared from large pools of plasma from more than 10,000 healthy blood donors (Sewell, W.A. & Jolles, S., 2002). It contains more than 95% immunoglobulin (Ig) G, trace amounts of IgA and IgM, as well as various amounts of other plasma components, such as CD4, CD8, HLA molecules, cytokines, and coagulation factors (Gelfand, E.W., 2006). When being prepared, Ig is purified by fractionation and chromatography, and stabilized with sugars or amino acids. It is then treated with solvents and detergents to inactivate viruses (Buchacher, A. & Iberer, G., 2006). To obtain a highly enriched IgG preparation, it is concentrated in a manner that would prevent loss of biological activity and minimize unwanted side effects, such as the formation of protein aggregates (Buchacher, A. & Iberer, G., 2006). However, as IVIG is produced by many different companies, the monomer and dimer content may vary between preparations, and up to 3% non-active polymers may be found. In addition, the compositions of the different IVIG preparations vary in the purity of the IgG preparation, pH, osmolarity, and sodium and sugar content (Gelfand, E.W., 2006).

Polyclonal anti-D immunoglobulins (anti-D) are prepared from human plasma of repeatedly immunized RhD^- donors (Kumpel, B.M., 2007). It is essentially a polyclonal IVIG enriched for anti-D antibodies. As with IVIG, steps are taken to eliminate bacterial and viral contamination during the manufacturing process.

1.6.2 Mechanism of Action of IVIG

IVIG has proved to be an effective therapy in a number of autoimmune diseases and inflammatory states, including chronic inflammatory demyelinating polyneuropathy, multiple sclerosis, Guillain-Barré syndrome, Lambert-Eaton myasthenic syndrome, myasthenia gravis, dermatomyositis, and some types of inflammatory arthritis (Nimmerjahn, F. & Ravetch, J.V., 2008). Although IVIG has become a common treatment for a variety of autoimmune diseases including ITP (Imbach, P. *et al.*, 1984, Bussel, J.B. & Hilgartner, M.W., 1984, Bussel, J.B., 1989), its mechanism of action is still unclear.

One of the earliest postulated theories on the mechanism of action of IVIG involves competitive inhibition of activating FcγRs on phagocytic macrophages by IVIG sensitized erythrocytes (Salama, A. *et al.*, 1983). Early studies supported this theory, showing that the clearance of radiolabeled red blood cells (RBCs) was delayed by IVIG administration (Fehr, J. *et al.*, 1982), implicating FcγRII/III in antibody-mediated elevation of platelet counts (Clarkson, S.B. *et al.*, 1986), and successfully inhibiting ITP in mice expressing the human FcγRIII, by a blocking antibody to this receptor (Samuelsson, A. *et al.*, 2001). However, evidence that contradicts the involvement of MPS blockade have recently emerged. For example, $F(ab')_2$ fragments of IVIG, which lack FcγR-binding ability, were shown to elevate platelet counts in ITP patients (Tovo, P.A. *et al.*, 1984), and IVIG was shown to be ineffective at treating ITP in FcγRIIB knockout mice (Samuelsson, A. *et al.*, 2001, Crow, A.R. *et al.*, 2003).

Recent studies implicating the inhibitory FcγRIIB receptor in the mechanism of action of IVIG have shown that FcγRIIB is required for the efficacy of small-molecular-weight (Siragam, V. *et al.*, 2005) IVIG-complexes in mice (Samuelsson, A. *et al.*, 2001, Crow, A.R. *et al.*, 2003).

Indeed, animals deficient in the FcγRIIB protein were no longer protected by administration of IVIG in mouse models of ITP, rheumatoid arthritis, and nephrotoxic nephritis (Samuelsson, A. *et al.*, 2001, Crow, A.R. *et al.*, 2003, Kaneko, Y. *et al.*, 2006, Akilesh, S. *et al.*, 2004). In addition, IVIG therapy resulted in the up-regulation of the inhibitory FcγRIIB on effector macrophages (Samuelsson, A. *et al.*, 2001, Kaneko, Y. *et al.*, 2006, Bruhns, P. *et al.*, 2003). However, consistent with the low affinity of FcγRIIB, IVIG does not directly interact with this receptor, and may exert its therapeutic effect through priming of dendritic cells (DCs). Specifically, Siragam, V. *et al.* (2006) have shown that IVIG-primed DCs from FcγRIIB knockout mice could adoptively transfer the therapeutic effect of IVIG to normal thrombocytopenic mice, suggesting that IVIG interacts with the activating FcγRs (such as FcγRIIIA), thereby priming DCs to directly or indirectly exert anti-inflammatory effects on phagocytic macrophages (Fig. 1.6.2). Curiously, emerging evidence indicates that large complexes of IVIG cross-linked via monoclonal IgG antibodies are able to ameliorate PIT independent of FcγRIIB expression (Bazin, R. *et al.*, 2006), suggesting that FcγRIIB may not be necessary for IVIG efficacy when large IgG complexes are involved. This postulation is also supported by an earlier finding, showing that large particulate immune complexes (mouse RBC + anti-RBC IgG) were able to ameliorate murine ITP independent of FcγRIIB expression (Song, S. *et al.*, 2005). Also, a previous study has suggested that autoantibody specificity may predict IVIG efficacy, as IVIG was effective at ameliorating GPIIb/IIIa antibody-induced but not GPIIbα antibody-induced PIT in a mouse model of ITP (Webster, M.L. *et al.*, 2006). This implies that IVIG may exert its effects by influencing Fc-dependent (anti-GPIIb/IIIa induced) platelet clearance, as it was generally ineffective at elevating platelet counts when Fc-independent antibodies (anti-GPIIbα) were used to induce PIT.

Another potential mechanism of IVIG action involves modulation of cytokine production. A range of pro- and anti-inflammatory cytokines may be modulated by IVIG, including interleukin (IL)-6, IL-8, tumor necrosis factor (TNF) α, IL-1 receptor antagonist (IL-1Ra) (Aukrust, P. *et al.*, 1994), and IL-10 (Cooper, N. *et al.*, 2004a). Also, recent findings from our laboratory showed that the levels of IL-11 are dramatically increased 6 hours post IVIG administration, correlating with an increase in reticulated platelets (Katsman, Y. *et al.*, 2008, Leontyev, D. *et al.*, 2009). Moreover, use of a neutralizing anti-IL-11 antibody dramatically inhibits the IVIG effect, suggesting that IVIG-induced production of the thrombopoietic cytokine, IL-11, plays an integral role in the mechanism of IVIG effect (Leontyev, D. *et al.*, 2009).

Additional ways in which IVIG may exert its therapeutic effect have been proposed, but at this point, the existing experimental evidence largely refutes their requirement for immediate IVIG efficacy. These mechanisms include neutralization of pathogenic antibodies by anti-idiotype antibodies present within IVIG preparations (Berchtold, P. *et al.*, 1989), immunomodulation by IVIG (such as enhancement of the suppressor T-cell function and decrease in autoantibody production (Delfraissy, J.F. *et al.*, 1985, Dammacco, F. *et al.*, 1986, Macey, M.G. & Newland, A.C., 1990)), inhibition of complement-dependent MPS effects (Mollnes, T.E. *et al.*, 1998) and clearance of cells (Basta, M. *et al.*, 1989), as well as induction of autoantibody-clearance via the neonatal Fc receptor (FcRn, Hansen, R.J. & Balthasar, J.P., 2002).

To summarize, although the exact mechanism of IVIG action is still elusive, it is clear that IVIG has a poly-specific nature, likely exerting its therapeutic effect via multiple mechanisms. The current prevalent models of IVIG action attribute its anti-inflammatory activity to the interaction of sialylated immunoglobulins with lectin-like receptors (SIGN-R1 or DC-SIGN) on splenic macrophages (Anthony, R.M. *et al.*, 2008), to soluble immune complexes binding to activating Fcγ receptors on dendritic cells (Siragam, V. *et al.*, 2006), or to production of the anti-

inflammatory cytokine, IL-11 (Leontyev, D. *et al.*, 2009). The first two mechanisms lead to a release of yet unidentified soluble mediators which down-regulate phagocytosis by possibly up-regulating inhibitory FcγRIIB receptors on macrophages.

Figure 1.6.2 Current paradigm of a possible IVIG mechanism of action

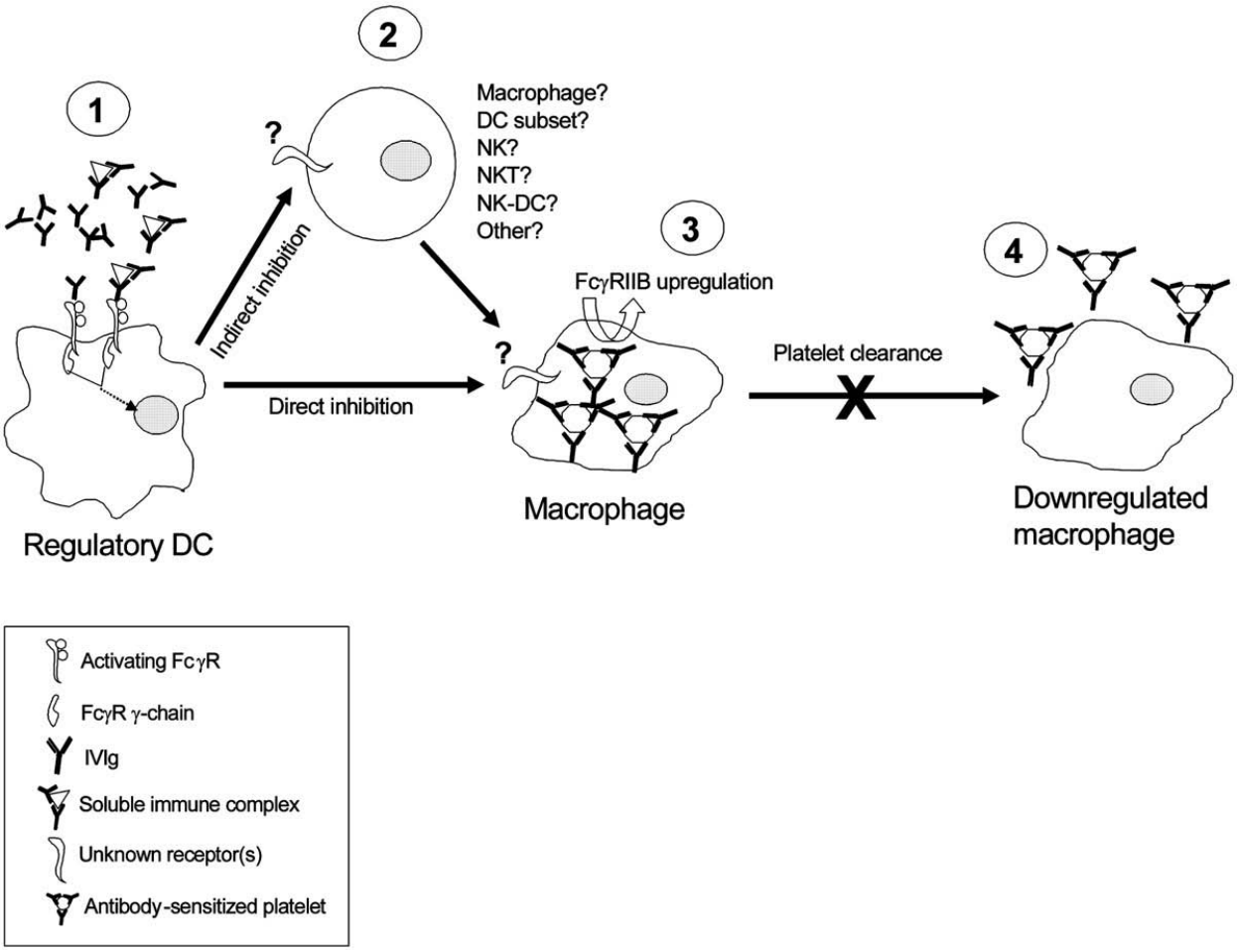


Figure 1.6.2 IVIG and soluble immune complexes (sICs) may inhibit autoimmunity by interacting with activating FcγRs on DCs in an FcγR γ-chain–specific manner and independent of FcγRIIB expression (step 1) (Siragam, V. *et al.*, 2006). It is proposed that the binding of IVIG or sIC to activating FcγRs on DCs drives an unknown signal that endows the DCs with the ability to regulate autoimmunity. These regulatory DCs may stimulate a secondary cell (indirect inhibition) via interaction with an unknown receptor (step 2), allowing them to then inhibit phagocytic macrophages by up-regulation FcγRIIB expression (step 3). Alternatively, the regulatory DCs may induce anti-inflammatory effects including up-regulation of macrophage FcγRIIB expression in step 3 (direct inhibition). The end result of either pathway is inhibition of sensitized platelet clearance by macrophages (step 4). Figure reproduced from *Transfusion Medicine Reviews*, 2008, 22(2):103-116. Copyright 2008 Elsevier Inc.

1.6.3 Mechanism of action of anti-D

The study of the mechanism of action of anti-D has been difficult, due to a lack of an appropriate animal model for its study. As mice do not express the D-antigen (Wagner, F.F. & Flegel, W.A., 2002), alternative approaches had to be developed for investigation of the mechanism of anti-D action. Currently, the best available approach is using monoclonal anti-erythrocyte antibodies, such as anti-TER-119 or M1/69, to mimic the effects of anti-D in a murine model of ITP (Song, S. *et al.*, 2003, Song, S. *et al.*, 2005). Despite the difficulties, the mechanism of anti-D action has been shown to be different from that of IVIG (Crow, A.R. *et al.*, 2003, Song, S. *et al.*, 2005, Bussel, J.B. *et al.*, 2001, Lazarus, A.H. & Crow, A.R., 2003). Most evidence supports the notion that anti-D works primarily by competitively inhibiting the MPS with antibody-coated RBCs (Salama, A. *et al.*, 1984, Bussel, J.B. *et al.*, 1991, Ambriz-Fernandez, R. *et al.*, 2002). For example, studies in the murine model of ITP have shown that anti-RBC antibodies down-regulate the expression of activating FcγRIIIA on phagocytic cells while increasing platelet counts (Song, S. *et al.*, 2005). Unlike IVIG, anti-D does not seem to function through FcγRIIB or regulate its expression (Song, S. *et al.*, 2005).

Anti-D may also exert its therapeutic effect by cytokine modulation, as was previously suggested for IVIG. Indeed, the cytokines induced by anti-D are somewhat different than those induced by IVIG, and include IL-6, IL-10, TNF-α, MCP-1 (Cooper, N. *et al.*, 2004a), IL-1Ra (Semple, J.W. *et al.*, 2002), as well as TGF-β and prostaglandin E2 (PGE_2) (Branch, D.R. *et al.*, 2006). However the role of these cytokines in the therapeutic activity of anti-D is still unclear. In this thesis I will provide additional evidence supporting the differential mechanism of action of IVIG and anti-D.

1.6.4 Disadvantages of Intravenous Immunoglobulins

The main disadvantage of IVIG therapy is its cost (Moler, F., 2001). With prices per gram of IVIG ranging from $50 to $100, and considering that the usual induction dose is 2g/kg, with maintenance therapy as required, the cost of IVIG therapy becomes prohibitive (Milgrom, H., 1998). Side effects associated with IVIG use are usually mild, affecting approximately 15 percent of patients, and include fevers, flushing, chest pain, muscle aches, headaches, shortness of breath and thrombosis (Hamrock, D.J., 2006).

Although anti-D is also acquired from human donations, it is a much cheaper therapy in comparison to IVIG, as it is effective at much lower doses (50-75μg/kg) (Cooper, N. *et al.*, 2004a). However, anti-D has its limitations, as it may only be administered to Rh-positive, pre-splenectomized patients. In addition, side effects associated with anti-D use include nausea, chills, and hemolytic anemia, which in some rare cases may be severe and potentially fatal (Olofinboba, K.A. & Greenberg, B.R., 2000, Levendoglu-Tugal, O. & Jayabose, S., 2001, Rewald, M.D. & Francischetti, M.M., 2004).

Not only are polyclonal IVIG and anti-D limited resources (Stiehm, E.R., 2000) due to their acquisition from human donations, but they also carry a potential for infectious disease transmission. Recipients of immunoglobulin therapy are at risk of contracting blood-borne diseases, particularly newly emerging infections, for which detection assays do not yet exist (for example variants of Crutchfeld-Jacob prions) (Milgrom, H., 1998).

1.6.5 Alternatives to Intravenous Immunoglobulins

Due to the limitations of the currently available treatments for ITP, ongoing research is aimed at developing alternative treatments. Some focus on developing cell-based therapies that would reduce the cost associated with IVIG use (Crow, A.R. & Lazarus, A.H., 2008), while others focus on developing humanized antibodies or agents that would target a specific process in the pathophysiology of ITP (such as inhibition of auto-antibody production or stimulation of TPO production, see section 1.5.3 for more details). However in our laboratory, we focus on the development of novel sulfur-containing small-molecular weight compounds targeted to inhibit FcγR-mediated phagocytosis by the MPS. Such compounds can potentially become alternatives to immunoglobulin therapies, as they would be inexpensive to produce, while eliminating the risk of disease transmission.

1.7 Small-Molecular-Weight Compounds as potential alternatives to immunoglobulin therapies

If certain drugs could be designed that were capable of elevating platelet counts in ITP patients, they would make great alternatives to immunoglobulin therapies. In addition to being significantly cheaper to produce, and therefore more available to patients, their use would also eliminate the risk of disease transmission. Consequently, our group set out to identify small-molecular weight compounds that may potentially exert therapeutic effects similar to those of immunoglobulin therapies.

As one of the possible mechanisms of immunoglobulin therapy action is competitive blockade of the activating FcγRs on MPS (Salama, A. *et al.*, 1983, Bussel, J.B. & Hilgartner, M.W., 1984, Bussel, J.B., 2000), therapeutic alternatives that affect the function of FcγRs may effectively inhibit phagocytosis. Early investigation into the development of such potential alternative therapeutics in our laboratory was based on reports that sulfhydryl (-SH) and/or disulfide (-S-S-) groups on macrophages (Mφ) play a role in Fcγ-mediated phagocytosis (Walker, W.S. & Demus, A., 1975, McKeown, M.J. *et al.*, 1984, Morgan, M.S. *et al.*, 1985).

Thimerosal is a preservative that is found in some gamma-globulin preparations, and was shown to inhibit FcγR-mediated attachment and phagocytosis *in vitro* (Rampersad, G.C. *et al.*, 2005). As thimerosal is an organomercuric (Hg_2^+) compound, its use resulted in significant toxicity to the cells at higher concentrations (Rampersad, G.C. *et al.*, 2005). However, its chemical structure has aided us in designing novel non-mercury-containing compounds which could have efficacy in inhibition of FcγR-mediated phagocytosis. As thimerosal can bind to free SH groups through its mercuric ion, it was surmised that compounds containing a disulfide bond, having the ability

to interact with free SH groups, may have similar effectiveness to that of thimerosal in inhibiting phagocytosis (Rampersad, G.C. *et al.*, 2005). Thus, a panel of novel sulfur-reactive chemical compounds containing SH or SS groups were designed and synthesized by Dr. Richard Langler (Chemistry Department in Mount Allison University, NB). Some of these compounds were tested using a monocyte monolayer assay (MMA) for efficacy of phagocytosis inhibition *in vitro* (Rampersad, G.C. *et al.*, 2005).

Small-molecular-weight molecules that can interact with free -SH or -S-S- groups on the cell surface of human Mφ were found to significantly inhibit the FcγR-mediated phagocytosis of antibody-coated human red cells *in vitro* (Rampersad, G.C. *et al.*, 2005). Particularly, the S-S compound *p*-nitrophenyl methyl disulfide (G-B), and the SH compound *p*-toluenesulfonylmethyl mercaptan (F-B) emerged as lead candidates due to their efficacy and lack of toxicity. These compounds were shown to inhibit phagocytosis by interfering with the binding of the Fcγ portion of the anti-D sensitizing RBCs to the FcγRs on the surface of Mφ (Rampersad, G.C. *et al.*, 2005).

Further examination of structural characteristics of the tested compounds revealed that the most efficacious compounds had benzene (phenyl) rings near reactive disulfide or free sulfhydryl groups (Rampersad, G.C. *et al.*, 2005). Also, the most critical structural requirement for *in vitro* chemically-mediated blockade of FcγR-mediated phagocytosis was found to be a disulfide bond, with efficacy enhancement provided by a *p*-nitrophenyl group (Foo, A.H. *et al.*, 2007). As disulfide groups covalently interact with free sulfhydryl groups, we hypothesized that any compound that interacts with free sulfhydryl groups has the potential to inhibit FcγR-mediated phagocytosis. For further design of potentially efficacious drugs for FcγR blockade, the optimal starting point was determined to be compounds containing a nitrophenyl ring structure and a disulfide linkage, such as G-B (Foo, A.H. *et al.*, 2007).

In this thesis, I am taking the next step in determining the efficacy of our candidate chemical compounds, by testing them in an *in vivo* mouse model of passive immune thrombocytopenia (PIT). In addition to the candidate compounds identified in our earlier work, I test a number of additional novel compounds synthesized by Dr. Langer, including 2,4-dinitrophenyl methyl disulfide (C7), and two sodium salts, *p*-nitrophenyl *ω*-hydrocarboxymethyl disulfide (C10) and *p*-nitrophenyl *ω*-hydrocarboxyethyl disulfide (C11). C7 was designed to contain two nitro groups, potentially enhancing the activity of the disulfide linkage, which might be activated by the nitro group. If so, the nitro groups may enhance the electrophilicity of the disulfide linkage leading to enhanced sulfenylation of naturally-occurring SH groups. The sodium salts C10 and C11 were designed for enhanced water solubility, by positioning an ionic group at the end of a non-aromatic disulfide substituent.

1.8 Mouse models of ITP

Mouse models of human ITP are employed as tools for the study of the underlying mechanisms of disease, for efficacy testing of potential novel therapeutics, and for discerning the mechanisms of action of available therapeutics. A range of mouse models have been developed for the study of ITP (McKenzie, S.E. & Reilly, M.P., 2004), including FcγR and FcγRIIB knockout mice, human FcγRIIA-expressing transgenic mice, as well as mice expressing human platelets (via stem-cell transplant) or humanized platelet antigens (such as GPIIb/IIIa).

All these models may be generally classified as either passive or active (McKenzie, S.E. & Reilly, M.P., 2004). In passive models, pathogenic antibody is injected into recipient animals, while in the active models the animal's own immune system generates the pathogenic antibodies spontaneously or as a result of induction by antigens. In an active model of ITP, hybrid male mice develop spontaneous autoimmunity due to a Y-linked antigen for accelerated autoimmunity that complements the polygenic predisposition to autoimmunity in NZW mice (Mizutani, H. *et al.*, 1993). One disadvantage of this model is that it is difficult to time when the mice might develop autoimmunity, and therefore, passive models of ITP are more commonly used.

There are two common approaches to passive immune thrombocytopenia (PIT) induction in mice. The first approach involves surgical implantation of an osmotic pump in the peritoneal cavity, which continuously delivers rat monoclonal anti-mouse platelet antibody directed against mouse platelet-specific integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa; MWReg30; CD41) at a rate of 0.04125μg per hour (Teeling, J.L. *et al.*, 2001, Deng, R. & Balthasar, J.P., 2007). The second commonly used method involves passive intraperitoneal injections of the same rat anti-mouse CD41 antibody at a rate of 2μg per day (Crow, A.R. *et al.*, 2001). Using either model, mice become

thrombocytopenic within 24 hours of initiation of the anti-CD41 due to immune mediated platelet destruction, and achieve low platelet counts over time. However the second model is technically easier to implement, as it does not require surgical intervention.

To test candidate compounds *in vivo*, we chose to use the widely employed mouse model of PIT induction described by Crow, A.R. *et al.* (2001). However, during our preliminary *in vivo* studies we were faced with a complication associated with this model (Foo, A.H., 2006). Although platelet counts dropped off dramatically within 24 hours of antibody administration and reached low counts that were maintained for a few days, platelet count began rising spontaneously starting on day 4 post initial anti-CD41 injection, despite continued daily anti-CD41 administration. As we needed a sensitive and consistent model that will allow us to discern subtle drug efficacy, we set out to investigate the cause of the platelet rebound in this mouse model. Our aim was to improve the current model of PIT for *in vivo* studies where maintenance of an anti-platelet antibody-induced thrombocytopenia as close to nadir as possible over an extended time period, is required.

1.9 Hypotheses

1. The observed platelet recovery seen after day 4 using a mouse model of PIT, is a result of a significant bone marrow response involving increased thrombopoiesis.

2. Antibody-induced thrombocytopenia will be maintained by counteracting the bone marrow response:
   a) Using sub-lethal doses of total body γ-irradiation (TBI) the bone marrow will be temporarily 'stunned', and its compensatory effect will be suppressed. This will maintain the antibody-induced thrombocytopenia, as long as the anti-platelet antibody is administered.
   b) By gradually increasing the concentration of the daily administered anti-platelet antibody, we will increase the amount of anti-platelet antibody available to induce clearance of the newly produced platelets, and counteract the bone marrow compensatory response.

3. One or more of the compounds synthesized by Dr. Langer, will show efficacy in the treatment of immune cytopenias.

1.10 Specific Objectives

1. To develop an improved mouse model of PIT that reproducibly maintains the antibody-induced thrombocytopenia at levels approaching nadir over time; thus, allowing for reliable investigation of different treatment modalities and their mechanisms of action.

2. To test small molecular weight compounds that have high solubility and low-toxicity, which would potentially be efficacious alternatives for treatment of immune cytopenias that currently utilize IVIG or anti-D therapies.

Chapter 2

Materials and Methods

2.1 Mice and Husbandry

Female outbred immunocompetent CD1 mice (6-8 weeks of age) were obtained from Charles River Laboratories (Montreal, PQ, Canada) or from Taconic Farms Inc. (Hudson, NY, USA). Inbred Balb/c and severe combined immunodeficient (SCID) CB.17 mice (8-12 weeks of age) were obtained from Charles River Laboratories (Montreal, PQ, Canada). Mice were kept under a natural light/dark cycle, maintained at 22 $\pm$ 4°C, and fed with standard diet and water *ad libitum*. All experiments were performed following animal-use protocols (AUP# 829.15) that were approved by the Toronto University Health Network Animal Research Committee (see Appendix IV for AUP).

2.2 Murine models of passive-immune thrombocytopenia (PIT)

2.2.1 Published model of PIT

PIT was induced and sustained by daily intraperitoneal (IP) injections of 68μg/kg monoclonal anti-platelet antibody (rat anti-mouse GPIIb) in 200μl phosphate-buffered saline (PBS) pH 7.2 exactly as previously described (Crow, A.R. *et al.*, 2001). The rat monoclonal anti-mouse platelet-specific integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa; clone MWReg30; rat IgG_1, κ) was purchased from BD PharMingen, Mississauga, ON. The dose of anti-platelet antibody was adjusted by mouse weight, so that CD1 and CB.17 SCID mice received 2μg/day, while Balb/c mice received 1.4μg/day.

2.2.2 Total Body γ-irradiation combination model

In the γ-irradiation combination model of PIT, mice were subjected to a single sub-lethal dose of total body γ-irradiation (TBI; 1 to 4 Gray (Gy)) from a Gammacell 40 Exactor (Nordion International Inc., Ottawa, ON), just prior to a first injection of 68μg/kg anti-CD41 (day 0). This was followed by daily anti-platelet antibody administration as described for the published model of PIT (Crow, A.R. *et al.*, 2001).

2.2.3 Dose escalation model of PIT

In the dose-escalation model of PIT, the dose of the monoclonal anti-platelet antibody was gradually increased over time as follows: mice received 68μg/kg on days 0 and 1, 102μg/kg on day 2, 136μg/kg on day 3, 170μg/kg on day 4, and 204μg/kg on day 5, raising the dose by 34μg/kg each day until the end of the experiment. When a modified version of the dose escalation model was used, mice received 68μg/kg on days 0 and 1, 102μg/kg on day 2, and 136μg/kg every day thereafter until the end of the experiment.

2.3 Blood collection

2.3.1 Method 1

This method of blood collection is a slight modification of the method previously described (Crow, A.R. *et al.*, 2001). 10µl (instead of 100µl) of whole blood were collected from the saphenous vein (instead of tail vein) of mice into capillary tubes preloaded with 10µl of 1% ethylene diamine tetraacetic acid (EDTA)/PBS. The blood was further diluted in 1% EDTA/PBS to a final dilution of 1:12,000 prior to platelet enumeration by FACS.

2.3.2 Method 2

Whole blood was collected on a daily basis by pipetting 10µl directly from the saphenous vein of mice into 990µl of 10% citrate-phosphate-dextrose-adenine (CPDA)/ PBS solution as suggested by Dr. John W. Semple (St. Michael's Hospital, Toronto, ON). The blood was further diluted in 10% CPDA/PBS to a final dilution of 1:1000 prior to platelet enumeration by FACS. Upon comparison of Methods 1 and 2, Method 2 became the method of choice, used in all experiments throughout this work.

2.4 Data collection and analysis

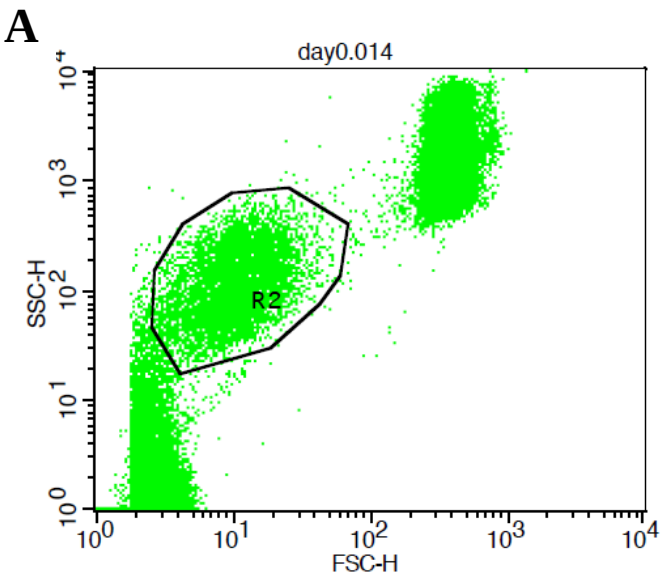
2.4.1 Platelet enumeration

Platelet counts were obtained by acquiring the blood samples collected by Method 2 on a calibrated Becton-Dickinson FACS Calibur for 2 minutes at medium speed. To consistently keep track of platelet counts on a daily basis, an acquisition and analysis template was set up. For this purpose, 50µl of 1:100 10% CPDA/PBS diluted whole blood reference samples were incubated with 5µl FITC-conjugated anti-CD41 (BD PharMingen, Mississauga, ON) for 30 minutes at room temperature in the dark. Just prior to acquisition on a FACS Calibur, the stained samples were further diluted in 10% CPDA/PBS to 1:1000.

The samples were first resolved on a forward scatter (FSC) versus side scatter (SSC) plot (Fig. 2.4.1A). Platelets were then detected on a FL1 versus SSC plot (Fig. 2.4.1C), and a gate was set around the fluorescent platelet population (R1). A second size plot (FSC versus SSC, Fig. 2.4.1D) was limited to show the cells in the R1 region only. A gate around the platelet population was drawn (R2) and overlaid onto the original size plot which resolves all the cell populations in whole blood. In that manner, subsequent blood samples do not need to be stained, as the platelet population will always fall within the R2 region.

Actual platelet counts per litre were obtained by multiplying the raw platelet counts (total events within the R2 gate over a 2 minute time period) by a factor of 0.04×10^9. This conversion factor was derived by comparing the raw platelet counts to those obtained on an electronic machine platelet counter (Beckman Coulter LH750 Hematology Analyzer, Fullerton, CA, USA) to ensure that accurate platelet enumeration was achieved using this approach.

Figure 2.4.1 FACS Acquisition and Analysis Template for Platelet Enumeration

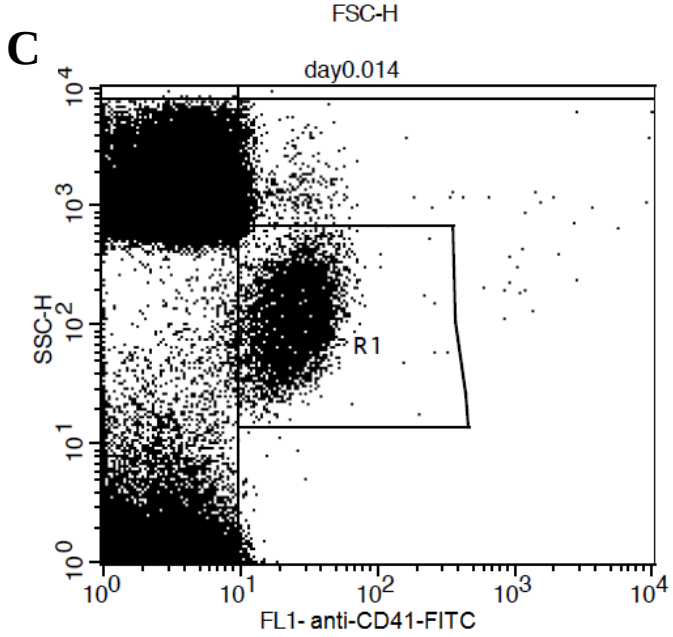


B

Region Statistics

File: day0.014
Gate: No Gate
Sample ID: 33 - FITC control
Total Events: 834390
X Parameter: FSC-H (Log)
Y Parameter: SSC-H (Log)

Region	Events	% Gated	% Total	X Mean	Y Mean
R1	25239	3.02	3.02	18.22	152.75
R2	26329	3.16	3.16	11.96	143.07



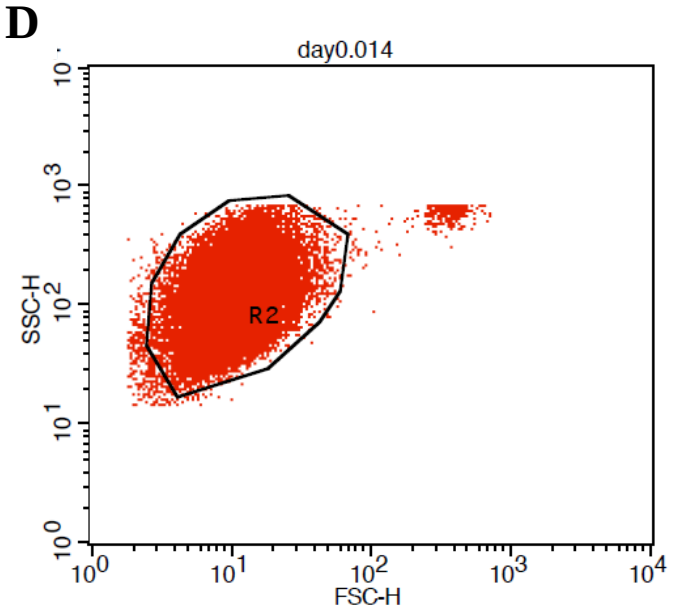


Figure 2.4.1 **A.** Size plot of FSC versus SSC showing the various populations observed in whole blood samples. The R2 gate indicates the platelet population as determined in the bottom two plots. The whole blood sample was incubated with FITC labeled anti-CD41 antibody.

B. Statistical analysis of the regions in this figure. Highlighted in orange are the total events acquired over a 2 minute time period. Highlighted in yellow are the raw platelet counts within the R2 gate. This raw count is later used to determine the actual platelet count.

C. A plot of FL1 versus SSC, differentiating between FITC fluorescent (to the right of 10^1 on the x-axis) and unstained cell populations (to the left of 10^1 on the x-axis). As anti-CD41-FITC was used to stain the whole blood sample, the fluorescent cell population is platelets. A gate (R1) encloses the platelet population.

D. A size plot of FSC versus SSC showing only the events within the R1 gate. A second gate (R2) is drawn around the platelet population, and is copied onto the first size plot. This ensures that the platelet population of further samples (even if unstained), will fall within the R2 gate on an FSC versus SSC plot of whole blood.

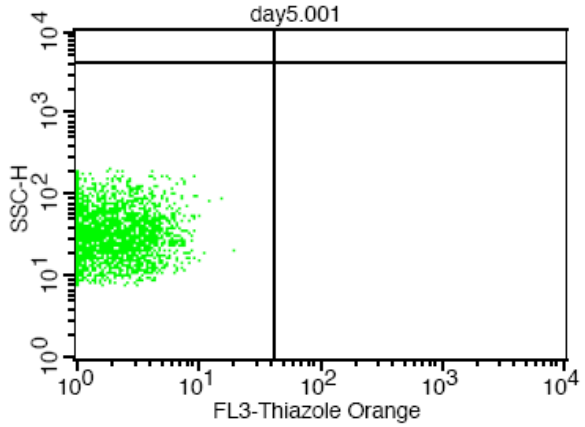
2.4.2 Enumeration of Reticulated Platelets

Young, newly produced platelets were identified by thiazole orange staining (Ault, K.A. *et al.*, 1992). Samples were prepared exactly as previously described (Bowen, D. *et al.*, 1991): a 1mg/ml stock solution of thiazole orange (TO) (Polysciences Inc., Warrington, PA, USA) was prepared in methanol, and diluted 1:10,000 just prior to use in PBS containing 0.002M EDTA and 0.02% sodium azide. 5µl of whole blood were pipetted directly from the saphenous vein of mice into 1ml of the TO solution, and incubated for at least 30 minutes at room temperature in the dark. The samples were acquired (10000 and 5000 platelets of control and test samples, respectively) on a calibrated Becton-Dickinson FACS Calibur.

Unstained controls were tested in all experiments (Fig. 2.4.2A), but as TO staining is only partially RNA specific, and other platelet components such as dense granular pools of nucleotides can cause a substantial amount of non-specific staining (Richards, E.M. & Baglin, T.P., 1995), we used TO-stained control samples to set the analysis markers. To differentiate the reticulated platelets from the general platelet population, the gate was set on the TO-stained controls to give <1% TO positive events (Fig. 2.4.2B) as described by Semple, J.W. *et al.* (1997). As can be seen in Fig. 2.4.2C, when reticulated platelet counts increase, a shift in fluorescence is clearly observed in the test samples. This shift is quantified as percentage of reticulated platelets, which is then multiplied by the total platelet count of the corresponding sample to give reticulated platelet count.

Figure 2.4.2 Template Setup for Reticulated Platelet Enumeration

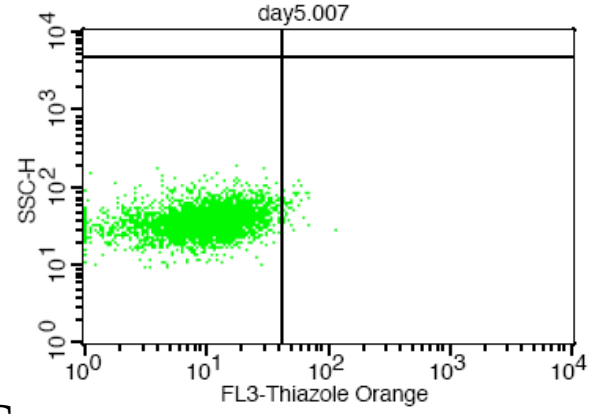
A



File: day5.001
Sample ID: unstained control
Gate: G2
Total Events: 123181

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	0	0.00	0.00	***	***
UR	0	0.00	0.00	***	***
LL	10978	100.00	8.91	1.91	40.37
LR	0	0.00	0.00	***	***

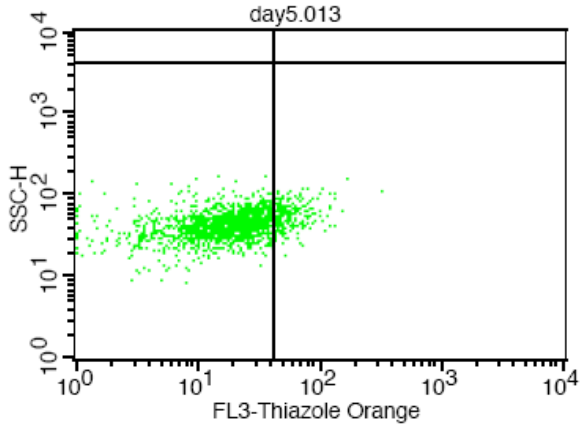
B



File: day5.007
Sample ID: TO control
Gate: G2
Total Events: 194131

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	0	0.00	0.00	***	***
UR	0	0.00	0.00	***	***
LL	11608	99.05	5.98	11.92	38.57
LR	111	0.95	0.06	51.31	59.12

C



File: day5.013
Sample ID: TO 2ug Ab
Gate: G2
Total Events: 164823

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	0	0.00	0.00	***	***
UR	0	0.00	0.00	***	***
LL	4862	85.63	2.95	18.47	44.30
LR	816	14.37	0.50	60.50	60.31

Figure 2.4.2 All three plots in this figure are FL3 versus SSC, and are limited to show platelet populations only (R2 region in Fig. 2.4.1). Percentages of Reticulated platelets on a given plot are highlighted in orange. These percentages are later used to calculate actual reticulated platelet counts.

A. Unstained sample from an un-manipulated control mouse. 10,000 events were acquired (i.e. 10,000 platelets), with all events falling under 10^1.

B. A Thiazole Orange stained sample from an un-manipulated control mouse. This sample was used to set the analysis marker to yield <1% positively stained platelets. 10,000 events were acquired (i.e. 10,000 platelets).

C. A Thiazole Orange stained sample from a test mouse that received 68μg/kg of anti-CD41 antibody on a daily basis. This particular sample was taken on day 5, when an increase in reticulated platelets was observed. As platelet counts in mice that receive anti-platelet antibody on a daily basis are very low, only 5,000 events were acquired (i.e. 5,000 platelets).

2.4.3 Data and Statistical Analysis

Total, as well as reticulated platelet counts are obtained on a daily basis over the course of the experiment (day 0 to day 6). Group means and standard deviations are determined and plotted. Student's 2-tailed t-test for equal variance is used to determine statistical significance ($p<0.05$) by comparing the platelet counts of test mice to the counts of control mice.

2.5 Treatments

The efficacy of various treatments was examined using either the TBI-combination or the dose-escalation model. IVIG and anti-TER-119 were used as positive controls for testing the new mouse models, while, a range of small molecular weight compounds were tested for their efficacy in treating PIT.

2.5.1 IVIG

A single dose (2g/kg) of human IVIG (Gammagard S/D, Baxter Healthcare Corporation, Glendale, CA, USA) was administered IP on day 2 of the experiment. As a 5% stock of IVIG was used, CD1 mice received 1ml, while Balb/c mice received 0.8ml of the preparation.

2.5.2 Anti-TER-119

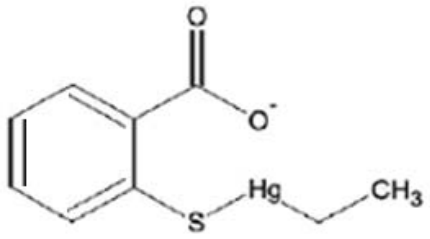
25μg of the purified rat-anti-mouse TER-119 (rat IgG_{2b}, κ, purchased from BD PharMingen, Mississauga, ON) per mouse, were administered IP on day 2 of the experiment in a total volume of 200μl PBS pH 7.2.

2.5.3 Small-Molecular-Weight Compounds

A range of commercial and newly synthesized compounds were tested for efficacy in treating PIT in the dose escalation mouse model (Fig. 2.5.3). Thimerosal was purchased from BioShop Canada Inc. (Burlington, ON), and *p*-nitrophenyl ethanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). The remaining chemicals were synthesized in the laboratory of Dr. Richard Langer, Mount Allison University (Sackville, NB). Drug design strategies included synthesis of similar compounds to those that already showed some efficacy *in vitro* (Rampersad, G.C. *et al.*, 2005), but having increased solubility and using exploitation of suspected effector moieties. The structures of the synthesized compounds that were tested for their efficacy (*p*-nitrophenyl methyl disulfide (G-B), *p*-toluenesulfonylmethyl mercaptan (F-B), 2,4-dinitrophenyl methyl disulfide (C7), *p*-nitrophenyl ω-hydrocarboxymethyl disulfide (C10) and *p*-nitrophenyl ω-hydrocarboxyethyl disulfide (C11)) are shown in Figure 2.5.3B, D-G. A list of additional compounds synthesized by Dr. Langer, but not tested in this work can be found in Appendix I.

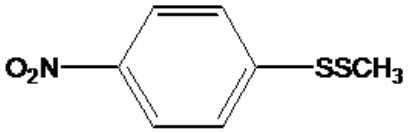
Figure 2.5.3 Chemical Structures of Tested Small-Molecular-Weight Compounds

A



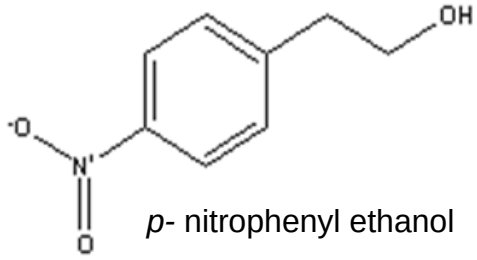
Thimerosal

B



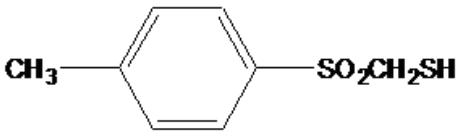
p- nitrophenyl methyl disulfide

C



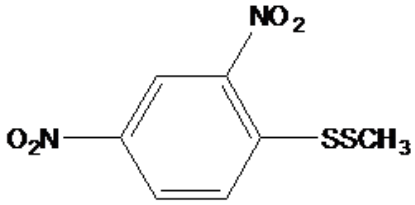
p- nitrophenyl ethanol

D



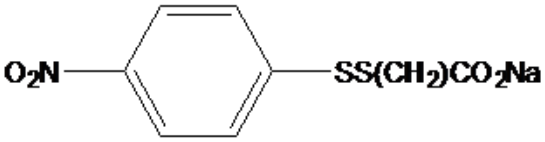
p- toluenesulfonylmethyl mercaptan

E



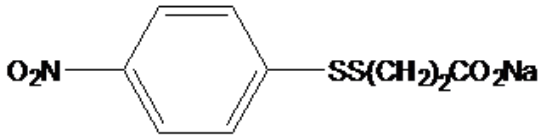
2,4- dinitrophenyl methyl disulfide

F



p-nitrophenyl ω-hydrocarboxymethyl disulfide

G



p-nitrophenyl ω-hydrocarboxyethyl disulfide

Figure 2.5.3 Structures of all chemical compounds tested in this work. **A.** Thimerosal is a lead compound identified in (Rampersad, G.C. *et al.*, 2005), and its structure formed the basis for modeling all other tested compounds. This chemical structure contains a mercury atom, which is not part of all other structures tested, due to its associated toxicity. Thimerosal is commercially available from BioShop Canada Inc. (Burlington, ON). **B.** *p*-nitrophenyl methyl disulfide is a first generation compound that was synthesized by Dr. Richard Langer, and is also a lead compound identified by (Rampersad, G.C. *et al.*, 2005). Its chemical structure forms the basis for modeling of second generation compounds, as its functional groups were implicated in the efficacy of phagocytosis inhibition (Foo, A.H. *et al.*, 2007). **C.** *p*-nitrohenyl ethanol is a commercially available compound that has desirable functional groups (phenyl and nitro), but lucks a disulfide bond, essential for phagocytosis inhibition *in vitro* (Foo, A.H. *et al.*, 2007). **D.** *p*-toluenesulfonylmethyl mercaptan is a first generation compound synthesized by Dr. Richard Langer that has previously shown efficacy in phagocytosis inhibition *in vitro* (Rampersad, G.C. *et al.*, 2005). It is structurally similar to G-B, but has a SH group instead of a disulfide bond. **E.** 2,4-dinitrophenyl methyl disulfide is a second generation polar compound, that in addition to its increased solubility, has all the desirable functional groups for phagocytosis inhibition as previously identified *in vitro* (Foo, A.H. *et al.*, 2007). **F. and G.** *p*-nitrophenyl ω-hydrocarboxymethyl disulfide and *p*-nitrophenyl ω-hydrocarboxyethyl disulfide are second generation sodium salts that were synthesized for increased solubility. They were both designed based on the structure of *p*-nitrophenyl methyl disulfide, and contain similar functional groups (phenyl, nitro, as well as disulfide bonds).

2.5.3.1 Maximum Tolerated Doses (MTDs)

The MTD refers to a dose level which may cause some signs of slight toxicity but not significant toxicities or mortalities (Harrison, P. *et al.*, 1997). The initial MTDs were performed by Nucro-Technics (Scarborough, ON, Canada), using a step-up and -down protocol in CD1 mice as described (Foo, A.H., 2006). The MTD for thimerosal was found to be 38.4mg/kg in 5% DMSO/PBS for male mice, and 76.8mg/kg in 10% DMSO/PBS for female mice (Branch, D.R., 2009). Also, the MTDs for *p*-nitrophenyl methyl disulfide and *p*-nitrophenyl ethanol were found to be 51.2mg/kg and 172.8mg/kg, respectively for both male and female mice, when administered intravenously in 10% DMSO/PBS.

As all other tested compounds are designed based on the chemical structure of *p*-nitrophenyl methyl disulfide, the MTDs of these compounds were determined by initially testing each compound at the MTD for *p*-nitrophenyl methyl disulfide (51.2mg/kg). If the compound showed no efficacy and no toxicity, the dose of the compound was doubled and re-tested. This step-up approach (Branch, D.R., 2009) was used under a UHN Animal Use Permit (AUP# 829.15, Appendix IV) until the compound reached its maximum solubility, or until toxicity to the mice was observed.

2.5.3.2 Compound Preparation, Solubility and Administration

Thimerosal was prepared fresh prior to use in either PBS or in PBS containing 10% DMSO (v/v), depending on the experiment. It dissolved well in either solvent at room temperature, and appeared as a clear liquid. 100µl of thimerosal were administered intravenously (IV) on day 2 of the experiment, 2 hours post anti-CD41 administration. As thimerosal was prepared at 11.2, 16.8 or 22.4mg/ml, the effective doses that the mice received were 38.4, 57.6 or 76.8mg/kg, respectively.

Stock solutions of all other chemical compounds were prepared in DMSO and stored at room temperature. Final dosing solutions were prepared in PBS containing 10% DMSO (v/v). Table 2.5.3.2 provides a comprehensive summary of the concentrations and solubility for all chemical compounds used in all experiments. Each row within the table represents the information for a given chemical compound used on a single group of mice (usually n=3 mice).

First generation compounds (*p*-nitrophenyl ethanol, *p*-nitrophenyl methyl disulfide and *p*-toluenesulfonylmethyl mercaptan) are very hydrophobic, and tend to precipitate out of solution even at very low concentrations. Although vortexing in combination with heating at 60C for 24 hours, were used to solubilise the chemicals as much as possible, the listed effective doses are likely underestimated as the chemicals sediment. Initially we administered the chemicals IV on day 2 of the experiment, 2 and 4 hours after anti-CD41 administration. But, to test the compounds at higher doses, we shifted to IP administration, which allows for testing of doses up to four times higher than by IV.

Second generation compounds were chosen for increased solubility due to either polarity or presence of a hydrophilic group. *p*-nitrophenyl ω-hydrocarboxymethyl disulfide and *p*-

nitrophenyl ω-hydrocarboxyethyl disulfide were both soluble at a lower concentration (1.28mg/ml), but went into suspensions at a slightly higher concentration (3mg/ml). The polar 2,4-dinitrophenyl methyl disulfide (C7) formed a uniform suspension at 3mg/ml, but precipitated, forming chunky sediment at 5mg/ml. Heating at 60C for 24 hours helped dissolve C7, forming a clear solution, but crystals of the compound coated the tube walls. All these compounds were administered IP to maximize the dose of chemical that is administered. However, due to the solubility problems, it is difficult to accurately estimate the effective doses received by mice.

Table 2.5.3.2 indicates the highest dose tested for each of the chemicals. Unfortunately, as all the chemicals precipitated out of solution at the highest concentration tested, there was no possibility of testing these chemicals for efficacy at higher doses.

Table 2.5.3.2 Candidate chemicals: Preparation, Solubility and mode of Administration

	Concentration	Administration	Effective dose	Preparation	Solubility
***p*-nitrophenyl ethanol**	17.7mg/ml	100µl x 2 IV	172.8mg/kg	RT*	Suspension with needle-like precipitate
***p*-nitrophenyl methyl disulfide (G-B)**	2.1mg/ml	100µl x 2 IV	20mg/kg	RT	Whitish suspension with some of the chemical precipitated
	5mg/ml	100µl x 2 IV	50mg/kg	Heating**	
	5mg/ml	800µl IP	200mg/kg	Heating	
***p*- toluene sulfonyl methyl mercaptan (F-B)**	1.75mg/ml	100µl x 2 IV	17.1mg/kg	RT	Whitish suspension with much of the chemical precipitated
	5mg/ml	100µl x 2 IV	50mg/kg	Heating	
	5mg/ml	800µl IP	200mg/kg	Heating	
2,4-dinitro phenyl methyl disulfide (C7)	1.28mg/ml	800µl IP	51.2mg/kg	Heating	Clear yellowish solution with some crystals on the tube walls
	5mg/ml	800µl IP	200mg/kg	Heating	Clear yellow solution with crystals on the tube walls
	3mg/ml	800µl IP	120mg/kg	RT	Milky yellow suspension
	10mg/ml	800µl IP	400mg/kg	RT	Milky yellow suspension with chunks of sediment
***p*-nitrophenyl *ω*-hydro carboxymethyl disulfide (C10)**	1.28mg/ml	800µl IP	51.2mg/kg	RT	Clear solution (dissolved)
	3mg/ml	800µl IP	120mg/kg	RT	White milky suspension
***p*-nitrophenyl *ω*-hydro carboxyethyl disulfide (C11)**	1.28mg/ml	800µl IP	51.2mg/kg	RT	Clear solution (dissolved)
	3mg/ml	800µl IP	120mg/kg	RT	White milky suspension with needle-like crystals forming

* RT = At Room Temperature

** Heating = 60^{o}C overnight + vortexing

Table 2.5.3.2 Each row is representative of compound characteristics and preparation used on a single group of mice in a given experiment. Conditions that are grouped by color for a given compound, were considered similar enough to be pooled together and represented as a single group in the results plots (Fig. 3.6.2 and 3.6.3).

Chapter 3

Results

3.1 Method of Blood collection

Initially, we attempted to use the published model of PIT (Crow, A.R. *et al.*, 2001) following the same method of blood collection (Table 3.1, Method 1). Unfortunately, our counts were inconsistent over time with large variability between individual mice. Following consultation with Dr. John Semple (St. Michael's Hospital, Toronto, ON, Canada), we changed our method of blood collection to Method 2 (Table 3.1). Using citrated anticoagulant, similar to that used in blood banks for platelet collection, instead of EDTA was found to result in less clumping, and the enumeration approach provided more accurate and consistent platelet counts.

The improvement using Method 2 over Method 1 is shown in Figure 3.1. Even when n=10 mice, there are relatively large standard errors for each point when Method 1 is used. In comparison, with Method 2 much tighter standard errors are obtained, with platelet counts in a given group of animals maintained within the normal range without much fluctuation over time. Consequentially, we decided to use Method 2 of sample collection and FACS analysis in all further experiments, with a slight modification. Instead of diluting the whole blood 1:100 prior to FACS analysis, we diluted whole blood samples to 1:1000, in order to prevent the flow rate of cells through the FACS machine from being adversely affected by the large numbers of cells. This method of platelet enumeration was validated by comparison to counts obtained with an electronic platelet counter (Beckman Coulter LH750 Hematology Analyzer, Fullerton, CA, USA).

Table 3.1 Comparison of two methods of blood collection and FACS analysis

	Method 1	Method 2
Diluent	1% EDTA	10% CPDA
Dilution factor	1:12,000	1:100*
Method of blood collection	10µl from the saphenous vein collected by a capillary tube, preloaded with 10µl of 1%EDTA	Pipetting 10µl with a p20 pipettor directly from mouse saphenous vein
Calculation of platelet counts from raw data	FACS platelet count x 12,000 dilution factor x 1,000,000µl/L / (24µl/min)	Multiply by a factor derived by comparing the FACS counts to counts from an electronic platelet counting machine (FACS platelet count x 0.04 conversion factor x 10^9/L)
Problems associated with this method	Very large variability between individual mice in the same group resulting in high standard deviations and inconsistencies in the data	Cells may not be resolved properly by the FACS at such high concentration, and may produce inaccurate counts*

* We use a dilution factor of 1:1000 so that the flow-rate of cells through the FACS machine is not hampered.

Figure 3.1 Comparison of Two Methods of Blood Collection

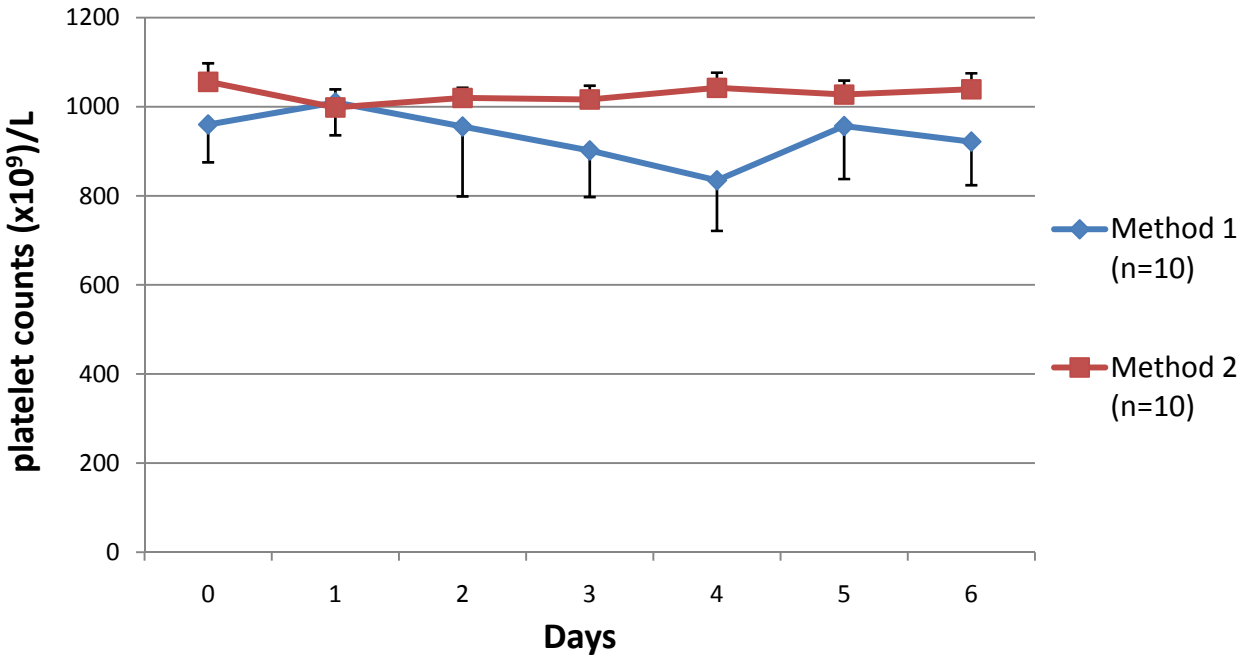


Figure 3.1 Mean Platelet counts in two groups of un-manipulated CD1 mice were obtained using methods 1 and 2 (see Table 3.1). n=10 mice in each group. + SEM are shown for method 1 and – SEM are shown for method 2.

3.2 The dose of anti-CD41 administered must be adjusted by mouse weight

When we attempted to test the published model of PIT (Crow, A.R. *et al.*, 2003) in Balb/c mice, by administering 2μg anti-CD41 antibody on a daily basis, we found that platelet counts drastically dropped (to 65.1 $\pm$ 10.5 x10^9/L) within 24hr, and remained at extremely low levels until day 4 (Fig. 3.2). It was apparent that the 2μg dose of anti-CD41 antibody was too high for the relatively small Balb/c mice. Not only did the platelet counts drop to extremely low levels, resulting in persistent bleeding following blood collection, but such high antibody concentrations made the mice sick (inactive and almost immobile). We found that in order to obtain comparable effects of anti-CD41 antibody in different mouse strains, its dose must be adjusted based on the mouse weight. As the average weight of Balb/c mice is 20.7 $\pm$ 0.6g (compared to 30 $\pm$ 1g for CD1 mice), we calculated the appropriate dose of anti-platelet antibody to be 68μg per 1kg of body weight, which is equivalent to 2μg/day in CD1 mice and 1.4μg/day in Balb/c mice.

Figure 3.2 The dose of anti-CD41 must be adjusted by mouse weight

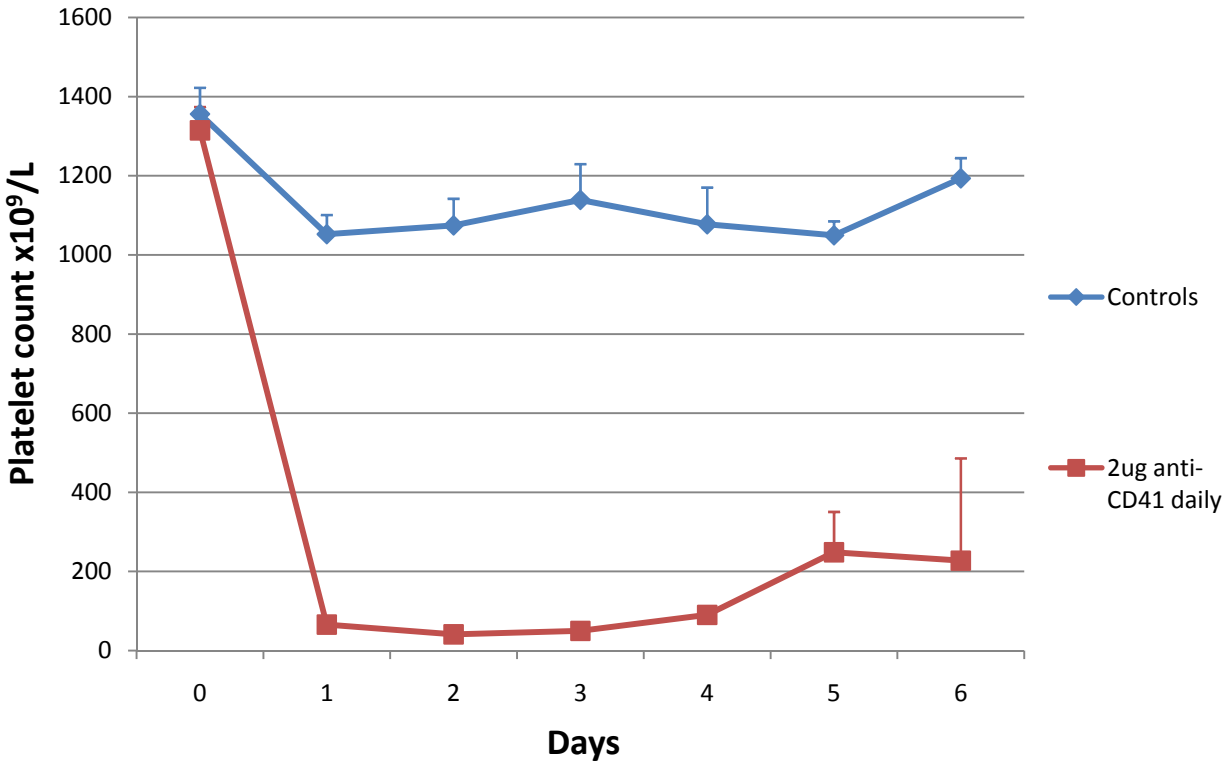


Figure 3.2 Platelet counts of Balb/c mice that received a daily injection of 2µg anti-CD41 per mouse as compared to unmanipulated controls. n=4 mice with bars representing standard deviations.

3.3 Spontaneous platelet recovery is a result of increased thrombopoiesis in the bone marrow

3.3.1 Immune response to anti-CD41 antibody is not the underlying cause of platelet rebound

We tested the published model (Crow, A.R. *et al.*, 2001) in three different mouse strains; outbred immunocompetent CD1 mice, inbred immunocompetent Balb/c mice and immunodeficient CB.17 SCID mice. We found that all mice regardless of strain tested become refractory to daily passive administration of anti-platelet antibody by day 4 (Fig. 3.3.1, 3.3.2A and C).

To rule out the possibility that the increase in platelet count may be a result of a mouse immune response to the rat-derived anti-platelet antibodies, we tested the model of PIT in CB.17 SCID mice. As these mice are devoid of B and T lymphocytes, they lack the ability to mount an immune response by producing antibodies. Immune thrombocytopenia was induced by passive daily administration of 2μg anti-platelet antibody, and platelet counts were followed for 4 days (Fig. 3.3.1 (Foo, A.H., 2006)). As with CD1 and Balb/c mice (see section 3.3.2), we observed that platelet counts in SCID mice increase significantly ($p=0.02$) on day 4 of the experiment in face of anti-platelet antibody administration. Thus, the observed platelet rebound was not a result of a mouse immune reaction to the rat derived antibodies.

Figure 3.3.1 Effect of daily anti-platelet antibody administration on platelet counts in CB.17 SCID mice

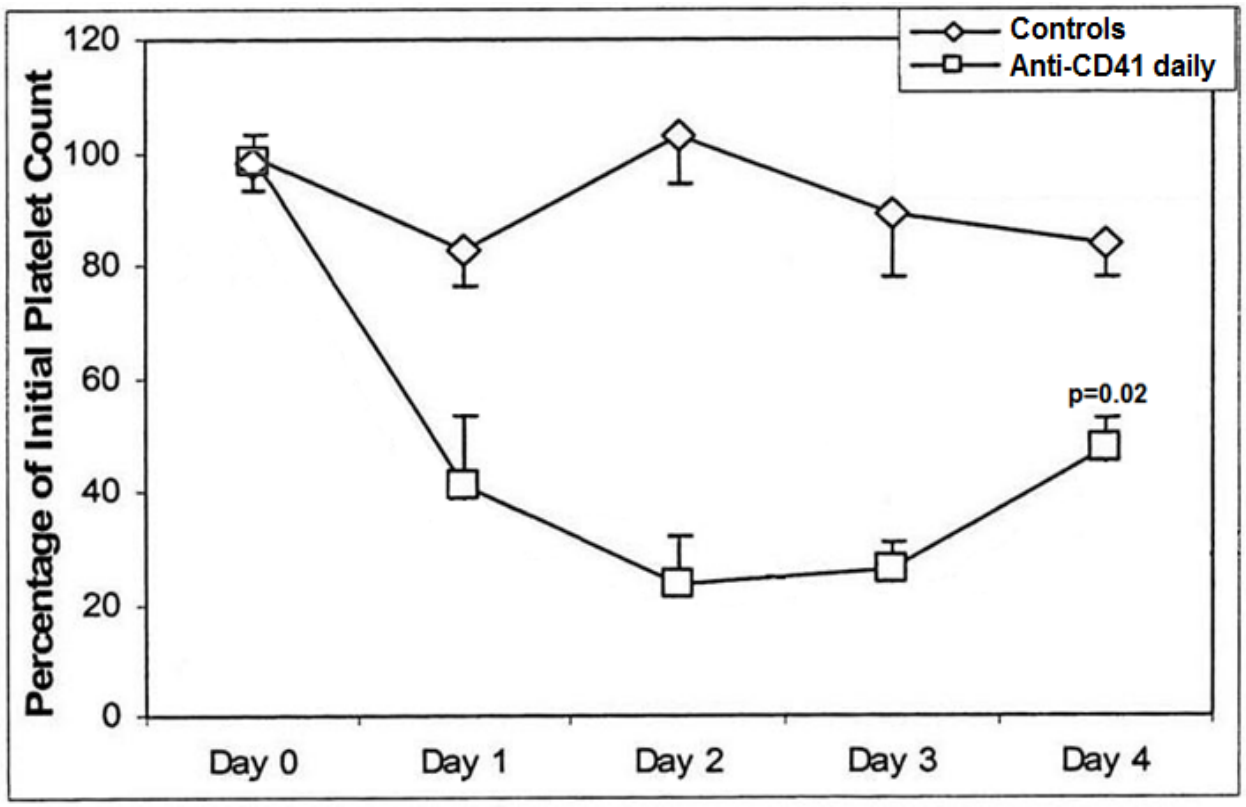


Figure 3.3.1 Figure reproduced with permission, from Foo, A.H. (2006). Percentages of initial platelet counts of CB.17 SCID mice (n=6 mice) are plotted over time in face of daily administration of 2μg anti-platelet antibody per mouse. Standard error bars with significant p value ($p<0.05$) for the difference in platelet counts between days 3 and 4 are shown.

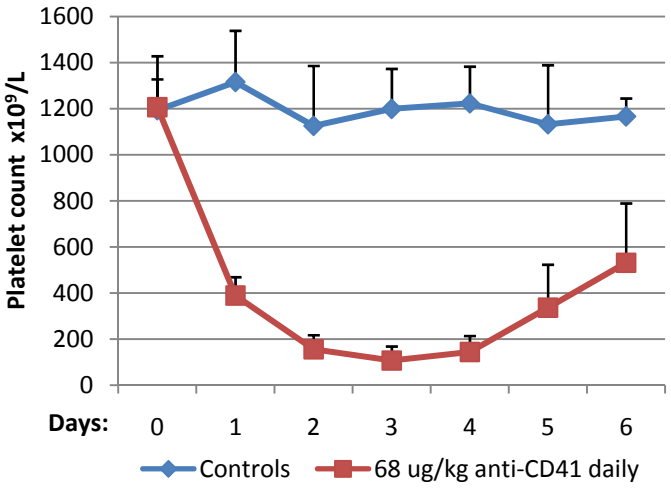
3.3.2 Spontaneous platelet recovery correlates with increased reticulated platelets counts in CD1 and Balb/c mice

Upon testing the published model of PIT (Crow, A.R. *et al.*, 2001) in outbred CD1 mice (Fig. 3.3.2A), we found that platelet counts drop to less than 200 $x10^9$/L by day 2, but increase significantly on days 5 and 6 of the experiment (336 $\pm$ 120 $x10^9$/L and 531 $\pm$ 150 $x10^9$/L, respectively). We hypothesized that the underlying cause of this increase in platelet counts was increased thrombopoiesis as a compensatory bone marrow response for anti-platelet antibody induced thrombocytopenia. To test this hypothesis, we used thiazole orange, which stains residual mRNA, to quantify the young platelets over time in face of daily anti-platelet antibody administration (Fig. 3.3.2B). The average number of reticulated platelets in un-manipulated CD1 controls was 15 $\pm$ 4.8 $x10^9$/L, whereas mice that received a daily dose of anti-platelet antibody showed a significant increase in the number of reticulated platelets on days 5 and 6 of the experiment (47 $\pm$ 19 $x10^9$/L and 37 $\pm$ 3.3 $x10^9$/L respectively). Notably, these increases directly correlated with the spontaneous increase in total platelet counts (Fig. 3.3.2A).

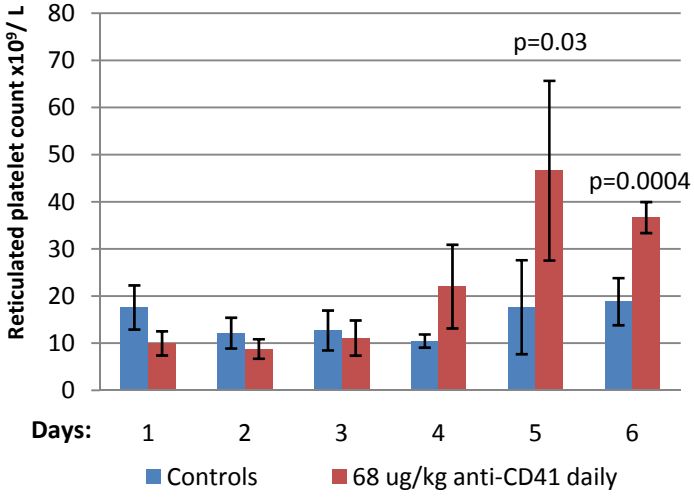
The results were similar, using inbred Balb/c mice. We found that total platelet counts increased from 49.9 $\pm$ 5.6 $x10^9$/L on day 2 to 606 $\pm$ 60 $x10^9$/L on day 5, and subsequently to within the normal range (812 $\pm$ 145 $x10^9$/L) on day 6 (Fig. 3.3.2C). Also, while the average number of reticulated platelets in un-manipulated controls was 12.3 + 1.6 $x10^9$/L, a significant increase was observed on days 4 to 6 of the experiment (33 + 11.5 $x10^9$/L, 63 + 15.5 $x10^9$/L and 21 + 6.3 $x10^9$/L respectively, Fig. 3.3.2C). Again, as was the case with CD1 mice, the increases in reticulated platelet counts directly correlated with the spontaneous increase in total platelet counts (Fig. 3.3.2D). These findings support the hypothesis that the observed increase in platelet counts is, at least in part, a result of increased thrombopoiesis resulting in a compensated thrombocytopenia.

Figure 3.3.2 Effect of daily anti-platelet antibody administration on total and reticulated platelet counts in CD1 and Balb/c mice

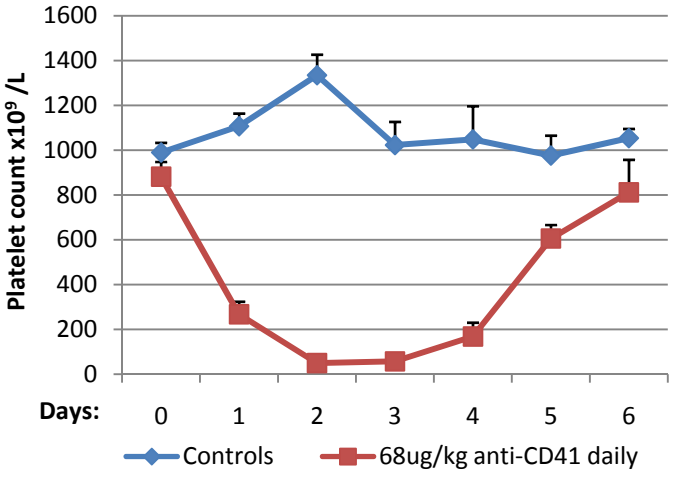
A – CD1 mice



B – CD1 mice



C – Balb/c mice



D – Balb/c mice

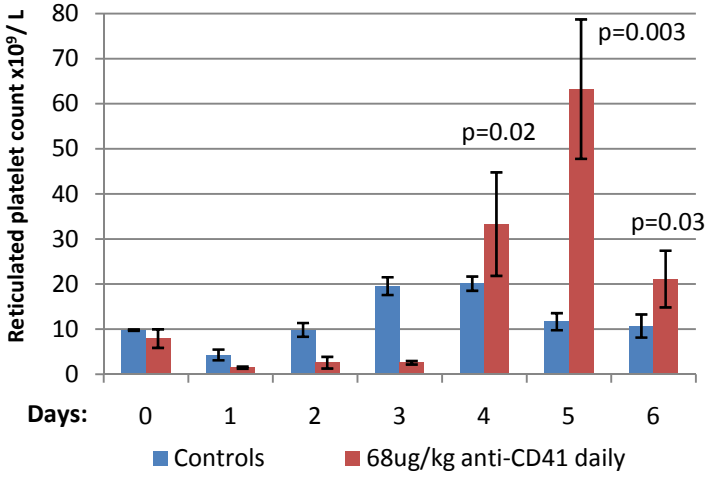


Figure 3.3.2 Kinetics of total and reticulated platelet counts over time in CD1 and Balb/c mice: **A.** Platelet counts of CD1 mice over time: unmanipulated controls compared to mice that received 68μg/kg (2μg/mouse) of anti-CD41 antibody daily. n=3 mice with bars representing standard deviations. **B.** Corresponding reticulated platelet counts of the CD1 mice shown in (A) as detected by thiazole orange staining and FACS analysis. p values of a 2-tailed t-test are for comparison to day 1 counts, with significant p values shown ($p<0.05$). **C.** Platelet counts of Balb/c mice over time: unmanipulated controls compared to mice that received 68μg/kg (1.4μg/mouse) of anti-CD41 antibody daily. n=3 mice with bars representing standard deviations. **D.** Corresponding reticulated platelet counts of the Balb/c mice shown in (C). p values of a 2-tailed t-test are for comparison to day 0 counts, with significant p values shown ($p<0.05$).

3.3.3 There is an abundance of available epitopes for anti-CD41 binding, with only 30% of platelets being bound by the anti-CD41 antibody at any given time

To test whether the percentage of anti-platelet antibody-coated cells changed over time, we incubated whole blood samples of CD1 mice with FITC-conjugated goat-anti-rat IgG (Serotec, NC, USA) for 30 minutes (on days 1, 4, and 5). We observed that the secondary antibody associated with 40 $\pm$ 0.4% of platelets from un-manipulated controls, and that this proportion remained unchanged over time (Fig. 3.3.3). As control mice have not received any anti-platelet antibody, this observation supports the fact that endogenous IgG sticks to platelets *in vivo* (Beardsley, D.S. & Ertem, M., 1998). Also, there were approximately 30% more FITC labelled platelets in the ITP group (67.4 $\pm$ 2.9% on average), a difference that can be attributed to anti-CD41 antibody binding to the platelet surface. The percentage of anti-CD41 bound platelets did not significantly fluctuate over time, suggesting that at any given time approximately 30% of platelets are bound by the anti-platelet antibody *in vivo*.

In another experiment we looked at whether the availability of epitopes for anti-CD41 binding on platelets decreased following anti-CD41 antibody administration. We incubated whole blood samples from control and anti-CD41-induced immune thrombocytopenic mice with FITC-conjugated anti-CD41 antibody on various days of the time course experiment (data not shown). We found that in both groups of mice, the anti-platelet antibody could bind to more than 99% of platelets. This finding suggests that the platelets of thrombocytopenic mice do not become saturated with the anti-CD41 antibody, despite daily antibody administration and also that anti-CD41 binds reticulated platelets as well as mature platelets as previously shown (Debili, N. *et al.*, 2001).

Figure 3.3.3 The proportion of platelets coated with rat anti-CD41 antibody does not change over time

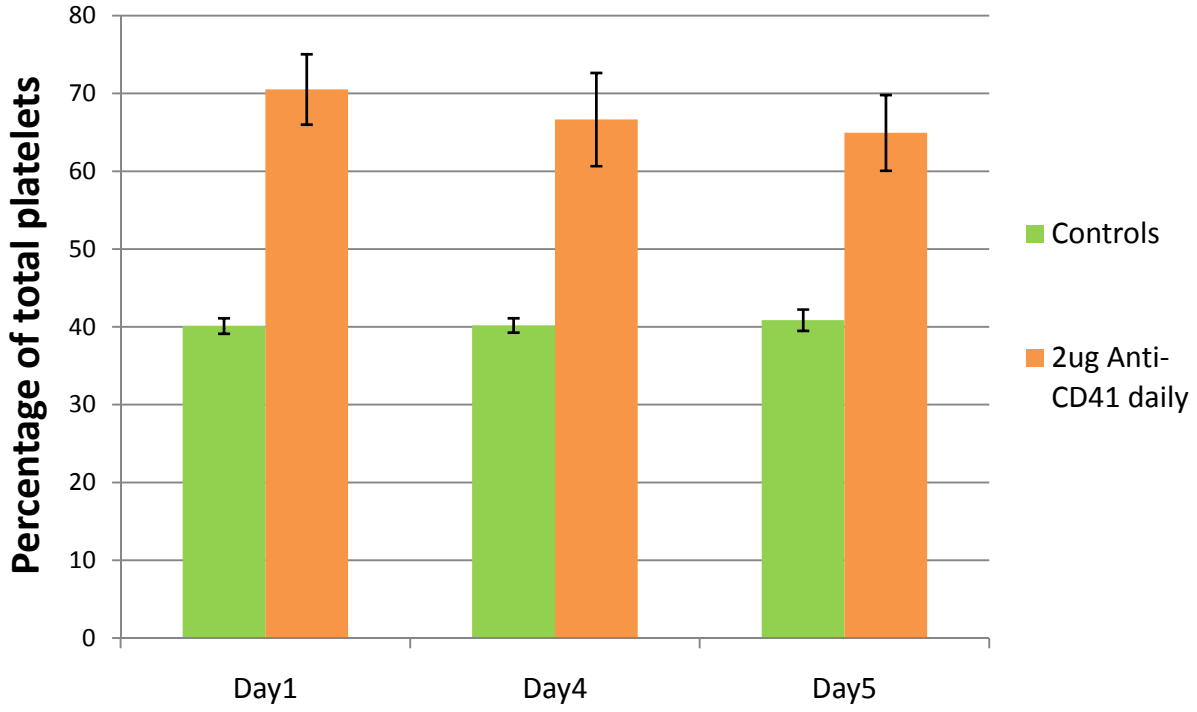


Figure 3.3.3 Percentage of platelets stained with a FITC labelled secondary antibody on days 1, 4 and 5. Whole blood samples from unmanipulated control CD1 mice (green), and from CD1 mice receiving daily injections of 2µg anti-CD41 (orange) were incubated with FITC labelled goat-anti-rat IgG. Fluorescently labelled platelets were detected and quantified by FACS. n=3 mice with bars representing standard deviations.

3.4 New mouse model of ITP #1: Sub-lethal total body irradiation in combination with passive daily anti-platelet antibody administration

3.4.1 TBI combination model effectively prevents the platelet rebound, maintaining consistently low levels of thrombocytopenia over the course of the experiment

As we found the recovery of platelets to be, at least in part, a result of compensated thrombopoiesis during daily anti-platelet antibody administration, we hypothesized that a sub-lethal dose of total body γ-irradiation (TBI) will suppress the bone marrow, thereby preventing thrombopoiesis and platelet rebound. To test this hypothesis, we first examined the effect of increasing doses of TBI alone (1Gy to 4Gy) on platelet counts. After monitoring platelet counts for 13 days, we found that suppression of platelet thrombopoiesis within the bone marrow was proportional to the dose of TBI, as measured by the lack of platelet production over time (Fig. 3.4.1A). We also observed that the TBI effect on platelet production became noticeable from day 6 post irradiation and onwards, consistent with the half-life of mouse platelets (Ault, K.A. & Knowles, C., 1995, Manning, K.L. & McDonald, T.P., 1997).

These findings suggested that passive daily anti-platelet antibody administration, in combination with a sub-lethal dose of TBI may maintain the antibody-induced thrombocytopenia, preventing bone marrow compensation. Upon comparison of this new model to the published model of immune-induced thrombocytopenia (Fig. 3.4.1B), we found that the new TBI-combination model was very effective at maintaining low platelet counts over the course of the experiment. In comparison, using the original mouse model without TBI, platelet counts began rising as

expected on day 4 of the experiment, and in this particular instance, returned to normal levels by day 5, despite daily administration of the anti-platelet antibody.

To demonstrate that TBI was in fact affecting the bone marrow and temporarily disrupting thrombopoiesis, we followed the reticulated platelet counts using this new combination model in CD1 mice (Fig. 3.4.1C). The average reticulated platelet count of un-manipulated controls was $8.9 + 3.2 \times 10^9$/L, while the average count of test mice was $8.1 + 3.1 \times 10^9$/L. No significant increase in reticulated platelet counts was observed at any point during the experiment, mirroring the lack of total platelet count increase during the course of the experiment (Fig. 3.4.1B).

Figure 3.4.1 Sub-lethal TBI mouse model of PIT

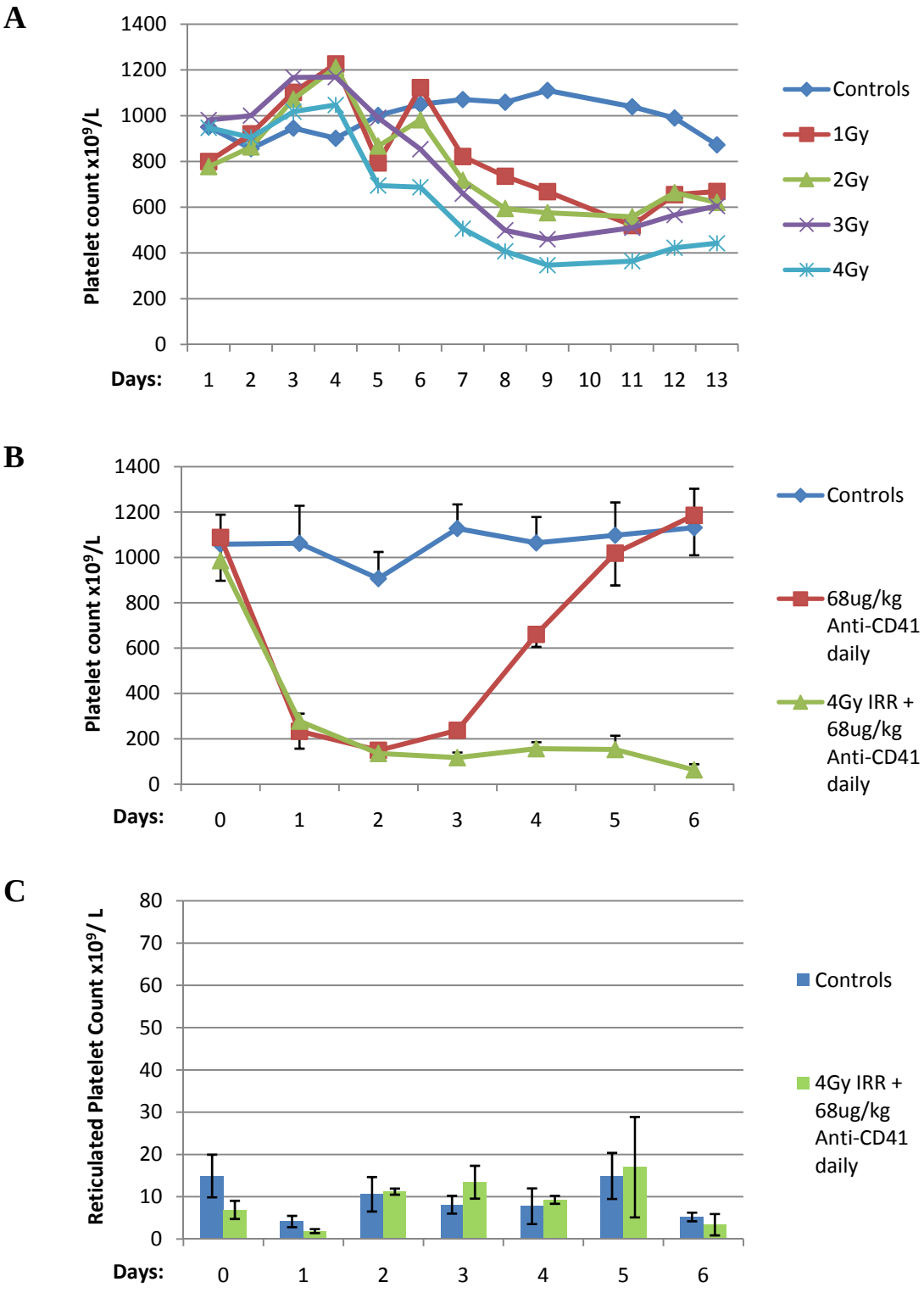


Figure 3.4.1 Platelet counts using the new TBI-combination mouse model of PIT:

A. A titration of sub-lethal TBI intensities and their effects on platelet counts in CD1 mice. Each group of mice (n=3 mice) was subjected to 1Gy, 2Gy, 3Gy, or 4Gy of total body γ-irradiation on day 0. Platelet counts were monitored daily by FACS. **B**. Comparison of the two mouse models of PIT. CD1 Mice in both test groups received 68μg/kg of anti-platelet antibody on a daily basis. Mice in the TBI group also received 4Gy of γ-irradiation on day 0. n=3 mice with bars representing standard deviations. **C**. Reticulated platelet counts do not rise when mice receive TBI in combination with a daily dose of anti-platelet antibody. The counts were done on the same samples used to plot total platelet counts in (B). Bars indicate standard deviations.

3.4.2 Daily administration of anti-CD41 antibody is necessary for continuous maintenance of antibody-induced thrombocytopenia

We have shown that daily administration of the anti-platelet antibody is imperative for maintaining the antibody-induced thrombocytopenia (Fig. 3.4.2). When the anti-platelet antibody was given only once, platelet counts dropped transiently (after 24 hours) and returned to normal levels. As the mice in this group were also subjected to 2Gy of γ-irradiation on day 0 of the experiment – platelet counts dropped again after day 6 due to bone marrow suppression by the irradiation. Note that in the group that received daily doses of anti-platelet antibody in conjunction with 2Gy γ-irradiation, platelet counts remained at low levels (around 200 $x10^9$/L). Interestingly, when a single dose of anti-platelet antibody was missed on day 6 of the experiment, platelet counts almost doubled (to 377 ± 40 $x10^9$/L) by the next day. Once anti-platelet antibody was again administered on day 7, platelet counts dropped back to low levels. Overall, our findings indicate that the TBI-anti-CD41 combination model is effective at maintaining antibody-induced thrombocytopenia throughout the course of the experiment, as long as anti-platelet antibody is administered on a daily basis.

Figure 3.4.2 Necessity of daily anti-platelet antibody administration

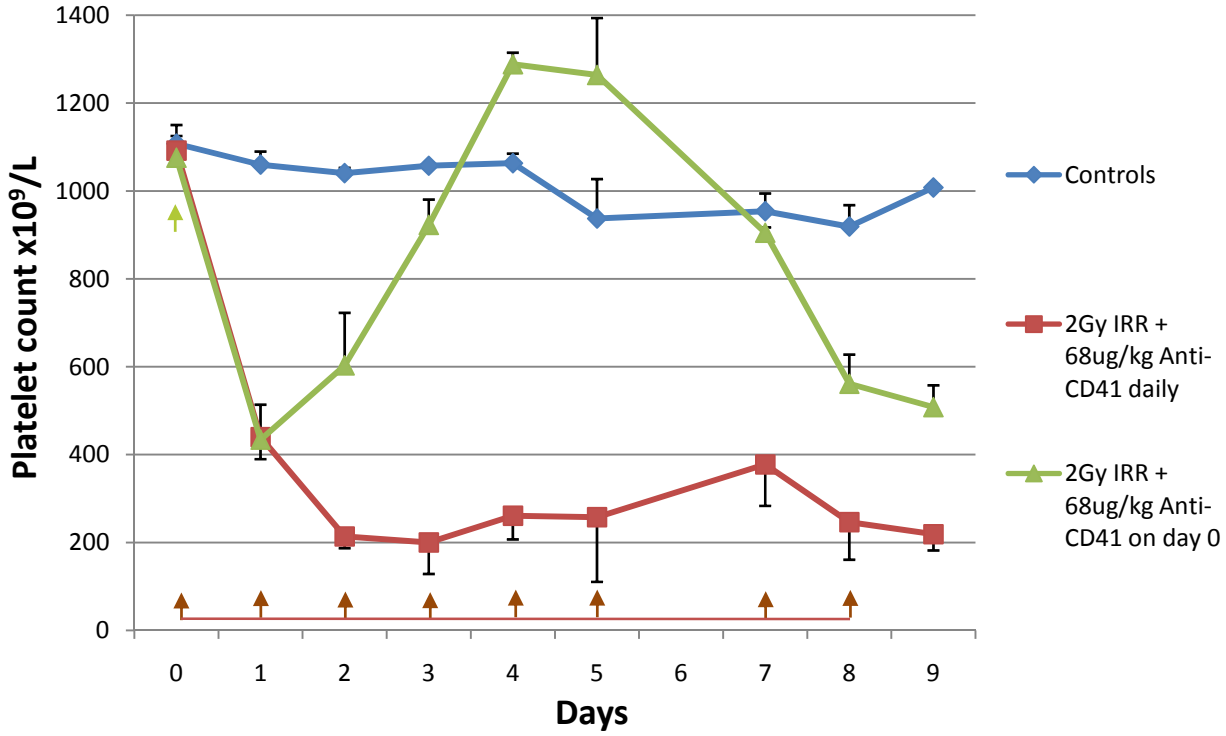


Figure 3.4.2 Importance of daily administration of anti-CD41 antibody. Mice in both test groups received 2Gy of γ-irradiation on day 0. Arrows indicate anti-platelet antibody administration. n=3 mice with bars representing standard deviations.

3.4.3 The TBI mouse model fails to respond to IVIG but responds to anti-TER-119 treatment

In the TBI-combination mouse model, using 2Gy or 4Gy of TBI, we found that administration of 2g/kg IVIG on day 2 does not show efficacy for improvement of the platelet count as has been previously shown using a PIT mouse model without TBI (Siragam, V. *et al.*, 2005). Indeed, treatment with IVIG resulted in a slight but statistically insignificant increase in platelet numbers on day 4 (Fig. 3.4.3A and B) that quickly returned to baseline levels. The fact that IVIG had a slightly better effect at the lower TBI dose (2Gy) demonstrates a dose-response although not significant in terms of increased platelet counts. Therefore, it appears that TBI prevents the efficacy of IVIG treatment in a dose dependent manner.

In addition, we have tested the efficacy of anti-TER-119, which works by a mechanism different from IVIG (Song, S. *et al.*, 2005). Anti-TER-119 was shown to be effective in significantly increasing platelet counts despite the high TBI dose being used (4Gy) in the TBI-combination mouse model (Fig. 3.4.3C). Mice that received 25μg of anti-TER-119 IP on day 2 of the experiment showed a significant increase in platelet counts on day 4 of the experiment (588 + 58 $x10^9$/L) when compared to controls (248 + 75 $x10^9$/L). The increase was sustained on day 5 as well (723 + 465 $x10^9$/L as compared to 131 + 23 $x10^9$/L), however, it was not statistically significant due to high variability. Overall, the therapeutic effect of anti-TER-119 was transient in nature, with platelet counts dropping on day 6. Together, these findings suggest that although TBI affects the thrombopoiesis in the bone marrow, the TBI combination model is useful for studying treatment modalities that work by a mechanism other than effects on bone marrow.

Figure 3.4.3 Anti-TER-119 treatment is effective but IVIG is ineffective when using the TBI-combination model

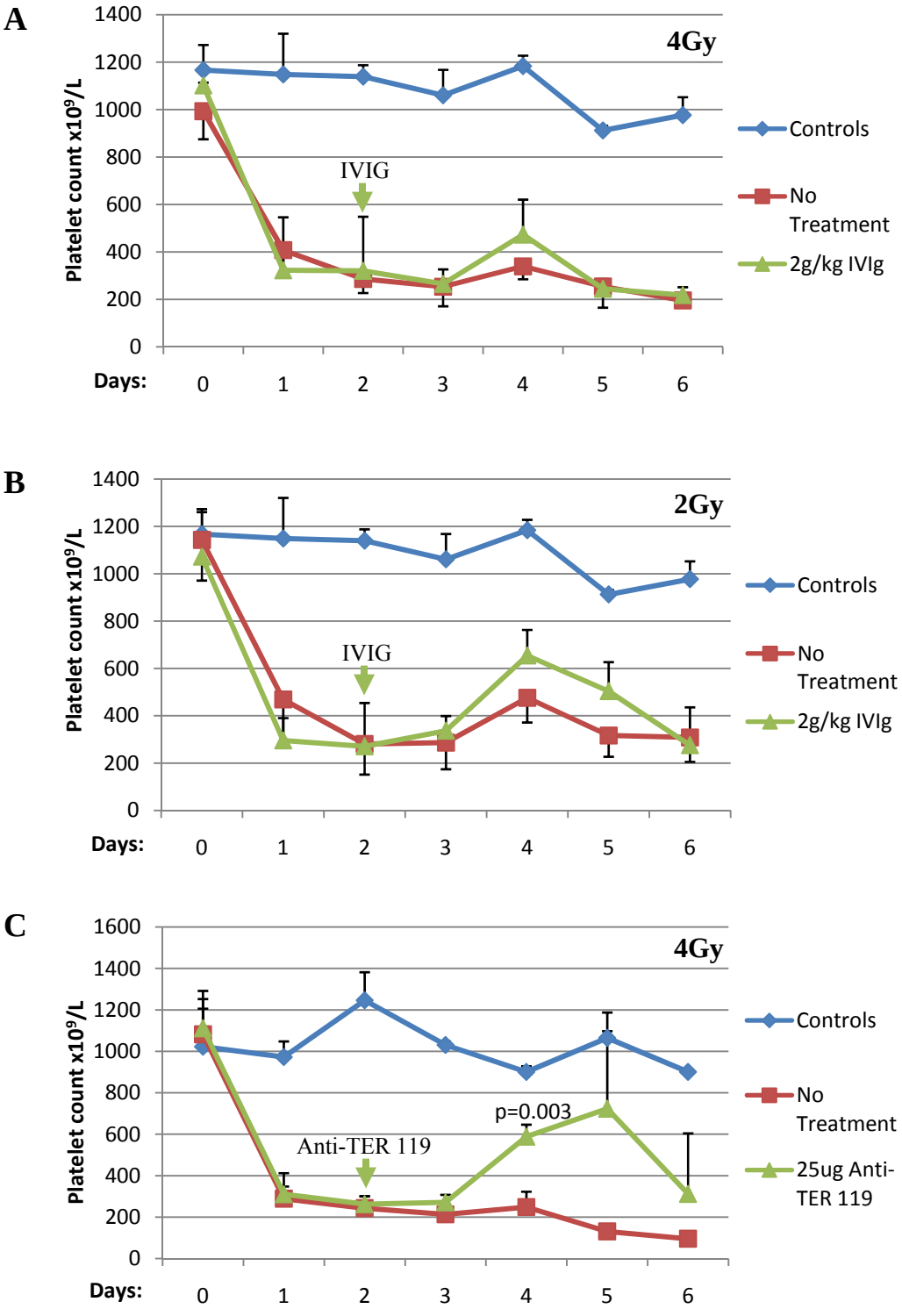


Figure 3.4.3 Efficacy of treatments using the TBI-combination model in CD1 mice:

A. All test mice received 4Gy TBI on day 0, as well as daily injections of 68μg/kg anti-CD41 antibody. Mice in the treatment group received 2g/kg of IVIG IP on day 2 of the experiment. n=3 mice with bars representing standard deviations. **B**. All test mice received 2Gy TBI on day 0, as well as daily injections of 68μg/kg anti-CD41 antibody. Mice in the treatment group received 2g/kg of IVIG IP on day 2 of the experiment. n=3 mice with bars representing standard deviations. **C.** All test mice received 4Gy TBI on day 0, as well as daily injections of 68μg/kg anti-CD41 antibody. Mice in the treatment group received 25μg of anti-TER-119 IP on day 2 of the experiment. n=3 mice with bars representing standard deviations. Significant p value ($p<0.05$) of a 2-tailed t-test for comparison to controls is shown.

3.5 New mouse model of ITP #2: Gradual escalation of the anti-platelet antibody dose over the course of the experiment

3.5.1 Dose escalation of anti-CD41 antibody effectively prevents the platelet rebound

Although the TBI-combination mouse model is effective for maintaining the antibody-induced thrombocytopenia, γ-irradiation interferes with IVIG efficacy in a TBI dose-dependent manner. Therefore, we decided to explore an alternative model. As thrombopoiesis increases in face of daily administration of anti-platelet antibody, the percentage of platelets that bind anti-CD41 does not increase; thus, we hypothesized that by gradually increasing the concentration of anti-CD41 over the course of the experiment we would sensitize more platelets, including the young reticulated platelets, thereby inducing increased clearance of platelets by the mononuclear phagocyte system and, thus, maintain a consistent thrombocytopenic state close to the nadir.

We tested a dose-escalation protocol where the dose of anti-platelet antibody was gradually increased over time (for CD1 mice, we administered 2μg on days 0 and 1 followed by 3μg, 4μg, 5μg and 6μg on days 2, 3, 4 and 5, respectively). The dose corresponds to an initial dose of 68μg/kg on days 0 and 1 followed by an increase of 34μg/kg each day. We found that the dose-escalation model was very effective in maintaining the antibody-induced thrombocytopenia as compared to the original (Crow, A.R. *et al.*, 2001) model of PIT (Fig. 3.5.1A). Platelet counts in mice that received 2μg anti-platelet antibody daily, increased dramatically on days 5 and 6 (514 $\pm$ 124 x10^9/L and 677 $\pm$ 230 x10^9/L, respectively). In contrast, platelet counts in mice that received escalating daily doses of anti-platelet antibody remained very low (107 $\pm$ 82 x10^9/L and 144 $\pm$ 114 x10^9/L, respectively).

We also tested the blood samples collected in this experiment for reticulated platelet counts over time by thiazole orange staining (Fig. 3.5.1B). Once again we observed that reticulated platelets dramatically increase by day 5 (62 ± 8 x10^9/L), in mice that receive a daily dose of 2μg anti-platelet antibody. Notably, reticulated platelet counts in this group decrease on day 6 (32 ± 11 x10^9/L), despite an increase in total platelet counts. This could be due to a feedback mechanism that decreases production of new platelets when platelet counts approach normal levels. In comparison, reticulated platelet counts remain very low over time in the dose-escalation group. This finding is consistent with total platelet counts remaining at low levels when using the dose-escalation mouse model.

Figure 3.5.1 Comparison of anti-CD41 dose-escalation to same-dose mouse model of PIT

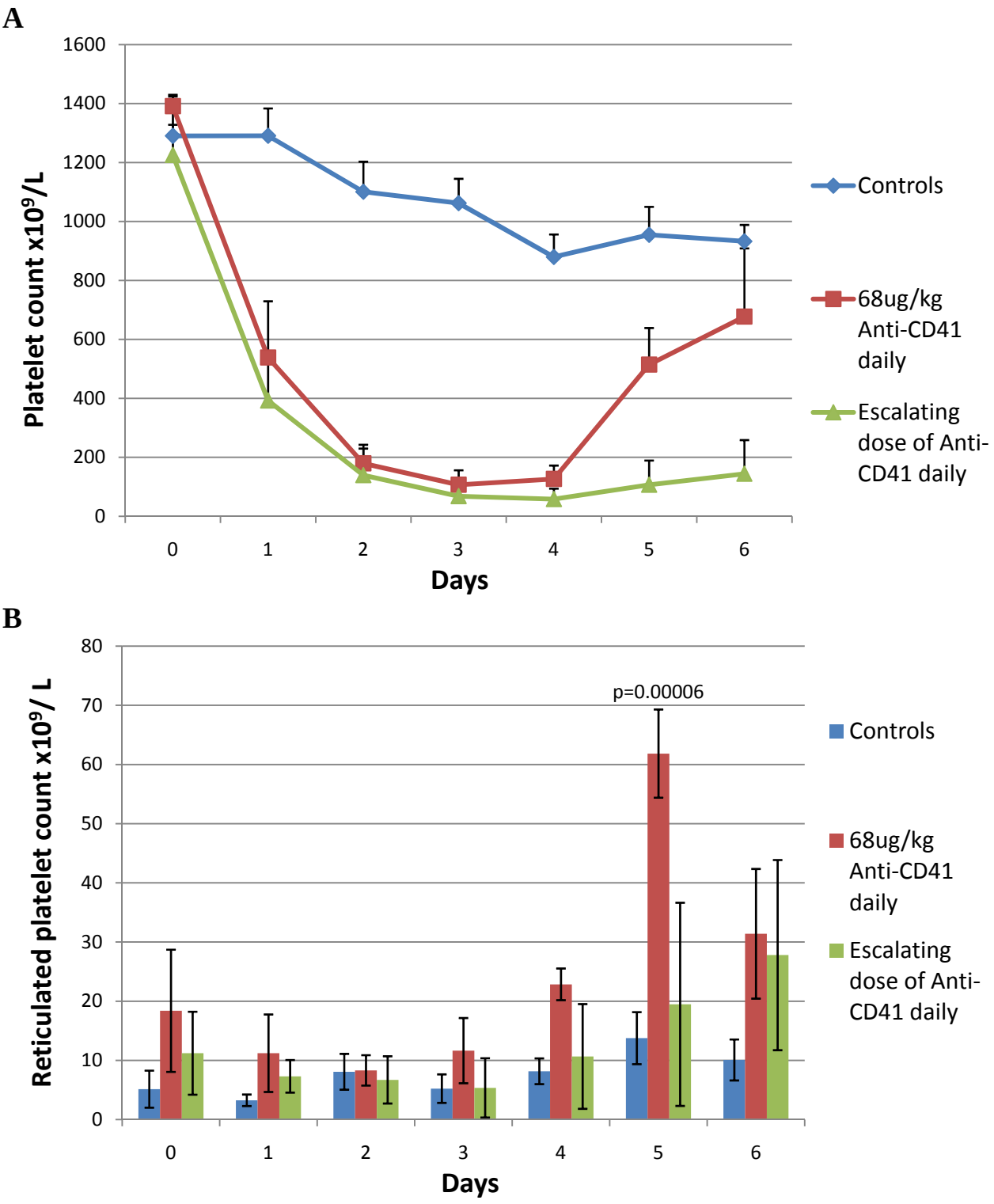


Figure 3.5.1. Kinetics of total and reticulated platelet counts using the new dose escalation mouse model of PIT in CD1 mice:

A. Plot of platelet counts over time in the anti-CD41 dose-escalation mouse model compared to mice that receive a daily single dose of anti-CD41 (68μg/kg). In the dose-escalation group, CD1 mice received 68μg/kg of anti-platelet antibody on days 0 and 1, followed by a daily increase of the antibody concentration by 34μg/kg (102μg/kg, 136μg/kg, 170μg/kg, and 204μg/kg on days 2, 3, 4 and 5, respectively). n=5 mice with bars representing standard deviations. **B**. Reticulated platelet counts as detected by thiazole orange staining and FACS analysis. Significant p value of a 2-tailed t-test for comparison to day 0 counts ($p<0.05$) is shown. n=5 mice with bars representing standard deviations.

3.5.2 IVIG is effective at increasing platelet counts using the dose-escalation mouse model

We next tested the effectiveness of treatment with IVIG to reverse the antibody-induced immune thrombocytopenia using the dose-escalation mouse model in both CD1 and Balb/c mice (Fig. 3.5.2). In the absence of treatment, using the dose escalation protocol, the platelet counts were maintained close to the nadir, at around 150 $x10^9$/L for CD1 and 60 $x10^9$/L for Balb/c mice. Notably, the standard deviations were much larger in the CD1 group due to their oubred nature. Unlike when using TBI (Fig. 3.4.3), IVIG was very effective in elevating platelet counts when using the dose escalation mouse model in both strains (Fig. 3.5.2A and B). In CD1 mice, although platelet counts did not reach the normal range, a significant increase in platelet counts was observed ($p=0.045$ and $p=0.009$, respectively) on days 4 and 5 (414 ± 82 $x10^9$/L and 546 ± 138 $x10^9$/L, respectively) following 2g/kg IVIG administration on day 2. Similarly, a significant increase in platelet counts ($p=0.02$, $p=0.003$ and $p=0.007$, respectively) was observed in Balb/c mice on days 3 to 5 (190 ± 72 $x10^9$/L, 555 ± 124 $x10^9$/L and 281 ± 71 $x10^9$/L, respectively) following IVIG administration on day 2.

Surprisingly, when monitoring reticulated platelets of Balb/c mice (Fig. 3.5.2C), we observed an increase in newly produced platelets only following treatment with IVIG that correlated with the transient nature of the IVIG effect on total platelet counts (Fig. 3.5.2B). This significant increase in total platelet counts starting 24 hours post IVIG administration and reaching a peak after 48 hours, correlated exactly with increased reticulated platelets, suggesting an effect of IVIG treatment on thrombopoiesis.

Figure 3.5.2 IVIG treatment shows efficacy in the anti-CD41 dose-escalation mouse model

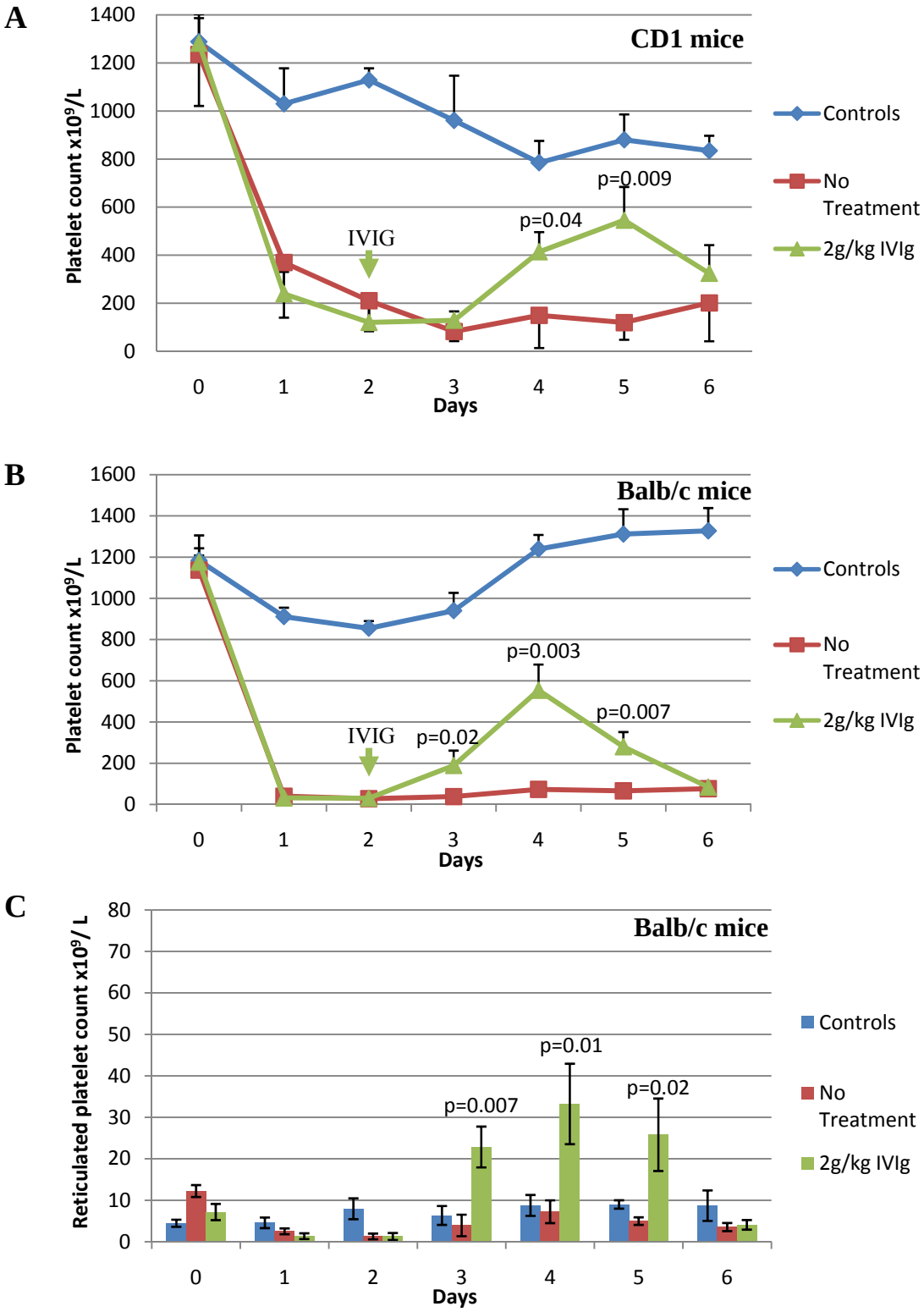


Figure 3.5.2 Efficacy of IVIG treatment using the dose escalation model of PIT:

A. Efficacy of IVIG in CD1 mice. All test mice received an escalating dose of anti-CD41 antibody on a daily basis starting at 2μg (68μg/kg) of anti-CD41 on days 0 and 1, followed by daily increase in the antibody dose (3μg, 4μg, 5μg and 6μg on days 2, 3, 4 and 5, respectively). The dose-escalation represents an increase of 34μg/kg each day starting on day 2. Mice in the treatment group received 2g/kg of IVIG on day 2 of the experiment. Significant p values of a 2-tailed t-test for comparison to day 0 counts ($p<0.05$) are shown. n=3 mice with bars representing standard deviations. **B.** Efficacy of IVIG in Balb/c mice. All test mice received an escalating dose of anti-CD41 antibody on a daily basis starting at 1.4μg (68μg/kg) of anti-CD41 on days 0 and 1, followed by daily increase in the antibody dose (2.1μg, 2.8μg, 3.5μg, 4.2μg and 4.9μg on days 2, 3, 4, 5, and 6, respectively). The dose-escalation represents an increase of 34μg/kg each day starting on day 2. Mice in the treatment group received 2g/kg of IVIG on day 2 of the experiment. Significant p values of a 2-tailed t-test for comparison to controls ($p<0.05$) are shown. n=3 mice with bars representing standard deviations. **C**. Reticulated platelet counts of the samples show in panel (B), as detected by thiazole orange staining and FACS analysis. p values of a 2-tailed t-test are for comparison to day 0 counts, with significant p values indicated ($p<0.05$).

3.5.3 Higher doses of anti-platelet antibody may adversely affect megakaryocytes in the bone marrow

An additional concern arose from observing the kinetics of the IVIG effect on platelet counts. As platelet counts dropped by day 6 of the experiment following a peak on days 4 and 5, it was possible that the high doses of anti-CD41 (>170μg/kg) administered at later days of the dose escalation protocol were adversely affecting megakaryocyte viability and/or function, resulting in decreased platelet production. This possibility was supported by reticulated platelet counts in this mouse model (Fig. 3.5.2C), which also dropped after reaching a peak on day 4, following IVIG treatment.

We tested this possibility by examining the efficacy of IVIG in a slightly modified dose-escalation protocol in Balb/c mice. On days 0 and 1 the mice received 68μg/kg, followed by 102μg/kg on day 2, and 136μg/kg of anti-CD41 antibody on day 3 and every day thereafter (Fig. 3.5.3). Using this modified protocol, platelet counts reached higher levels following 2g/kg IVIG administration than when using the original dose escalation protocol (694 ± 175 x10^9/L compared to 555 ± 124 x10^9/L on day 4 and 693 ± 90 x10^9/L compared to 281 ± 71 x10^9/L on day 5) suggesting that the original dose-escalation protocol may result in some toxicity to megakaryopoiesis. Notably, as low platelet counts were consistently maintained using this modified protocol, it is likely more sensitive for monitoring efficacy of current and potential therapeutics. However, this approach did not provide a clear answer as to whether the megakaryocytes were adversely affected by the higher doses of anti-CD41 antibody, and a different experimental approach must be taken to answer this question. Indeed, as with the higher dose escalation model, platelet count dropped back to low levels by day 6. However, this transient effect may be related to a transient effect in the bone marrow induced by IVIG.

Figure 3.5.3 IVIG efficacy using a modified dose-escalation protocol

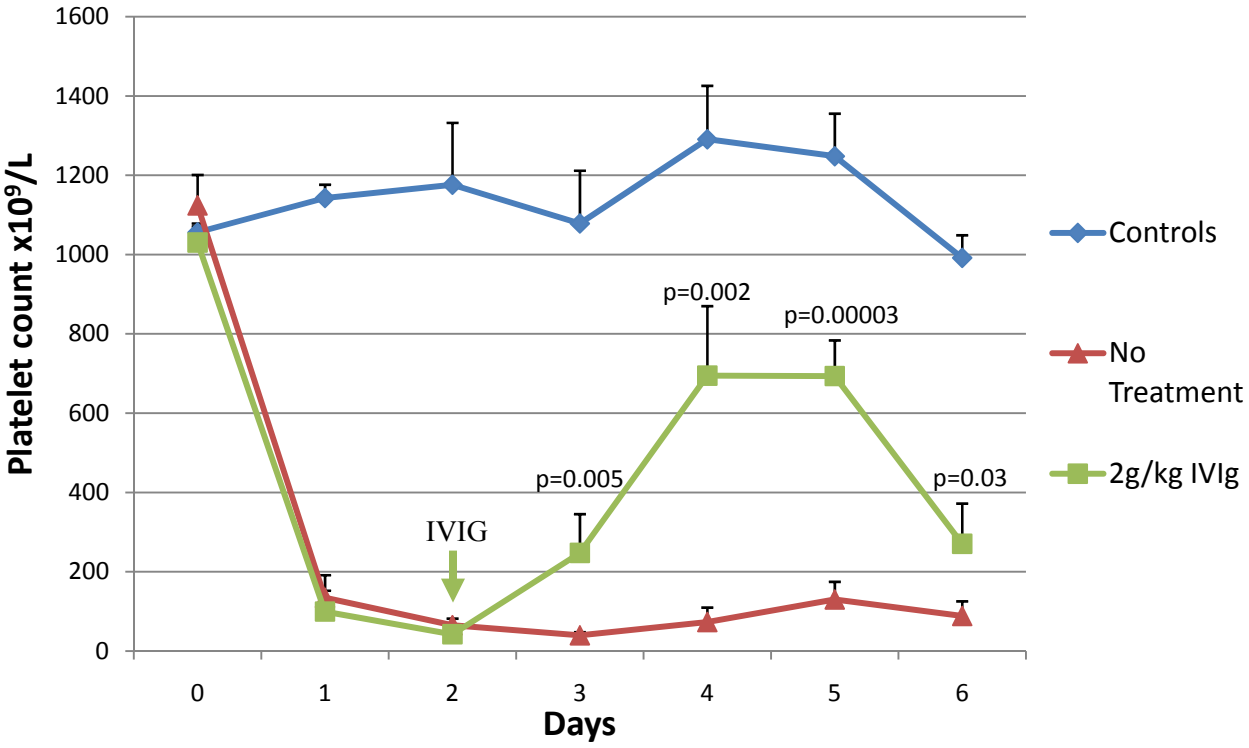


Figure 3.5.3 Platelet count kinetics in Balb/c mice using a modified dose escalation protocol. All test mice received 1.4µg (68µg/kg) of anti-CD41 on days 0 and 1, followed by 2.1µg on day 2 and 2.8µg on days 3,4 and 5. Mice in the treatment group received 2g/kg of IVIG IP on day 2 of the experiment. n=4 mice with bars representing standard deviations. p values of a 2-tailed t-test are for comparison to 'no treatment' controls, with significant p values indicated ($p<0.05$).

3.6 The efficacy of small-molecular-weight compounds *in vivo*

3.6.1 Thimerosal has no efficacy in elevating platelet counts in both the TBI-combination and the dose escalation models of PIT

The aim of this thesis was to design a consistent and sensitive mouse model of ITP in order to test candidate small-molecular-weight compounds as potential alternatives to IVIG treatment. Thimerosal is a chemical compound that was tested first, as it showed high efficacy in inhibiting phagocytosis *in vitro* (Rampersad, G.C. *et al.*, 2005). While toxic at high concentrations, it was tested in both of our mouse models at its determined maximum tolerated dose (MTD = 38.4mg/kg in 10% DMSO/PBS). Unfortunately, no significant efficacy was detected *in vivo* using either the TBI-combination or the dose escalation mouse model (Fig. 3.6.1).

When thimerosal was administered IV at its MTD on day 2 of the experiment to CD1 mice using the TBI-combination model, a slight increase in platelet counts was observed in the treatment group on day 4 (Fig. 3.6.1A). This increase is statistically significant when compared to day 3 values within the treatment group ($p=0.008$), but borderlines on significance when compared to untreated controls on day 4 ($p=0.051$). It is difficult to conclusively assess the effect of thimerosal in this model as the experiment was performed a single time at the early stages of investigation, when human error resulting from unrefined technical tail injection skills, could have been a factor.

Since the dose escalation model was adopted as the model of choice for testing candidate chemical compounds, thimerosal was tested in numerous experiments using this model. Balb/c female mice were used to test thimerosal at its MTD for male mice (38.4mg/kg) and at 2x the

MTD for male mice (76.8mg/kg), but within the MTD for female mice (Fig. 3.6.1B). CD1 female mice were used as well to test thimerosal at 38.4mg/kg (Fig. 3.6.1C), 1.5x (57.6mg/kg) and 2x (76.8mg/kg) the MTD for male mice (Fig. 3.6.1D). We found that thimerosal made the mice quite sick when administered IV at 38.4mg/kg and 57.6mg/kg. Their visible symptoms included ruffled fur, lighter and opaque eyes, cold extremities, invisible veins, lighter (anemic) blood, and general inactivity and indifference. When administered at 76.8mg/kg, thimerosal proved to be lethal by day 4 of the experiment in both strains (all CD1 mice in this group died by day 4, while one mouse per day was found dead in the Balb/c group following thimerosal administration on day 2). Unfortunately, in addition to its adverse effects, thimerosal showed no efficacy in elevating platelet counts in either mouse strain using this model. It is likely that thimerosal's toxic effects *in vivo* are masking its proven *in vitro* efficacy in inhibiting phagocytosis and potential for elevation of platelet counts in PIT.

Figure 3.6.1 Thimerosal efficacy using the TBI-combination and the dose escalation models of PIT

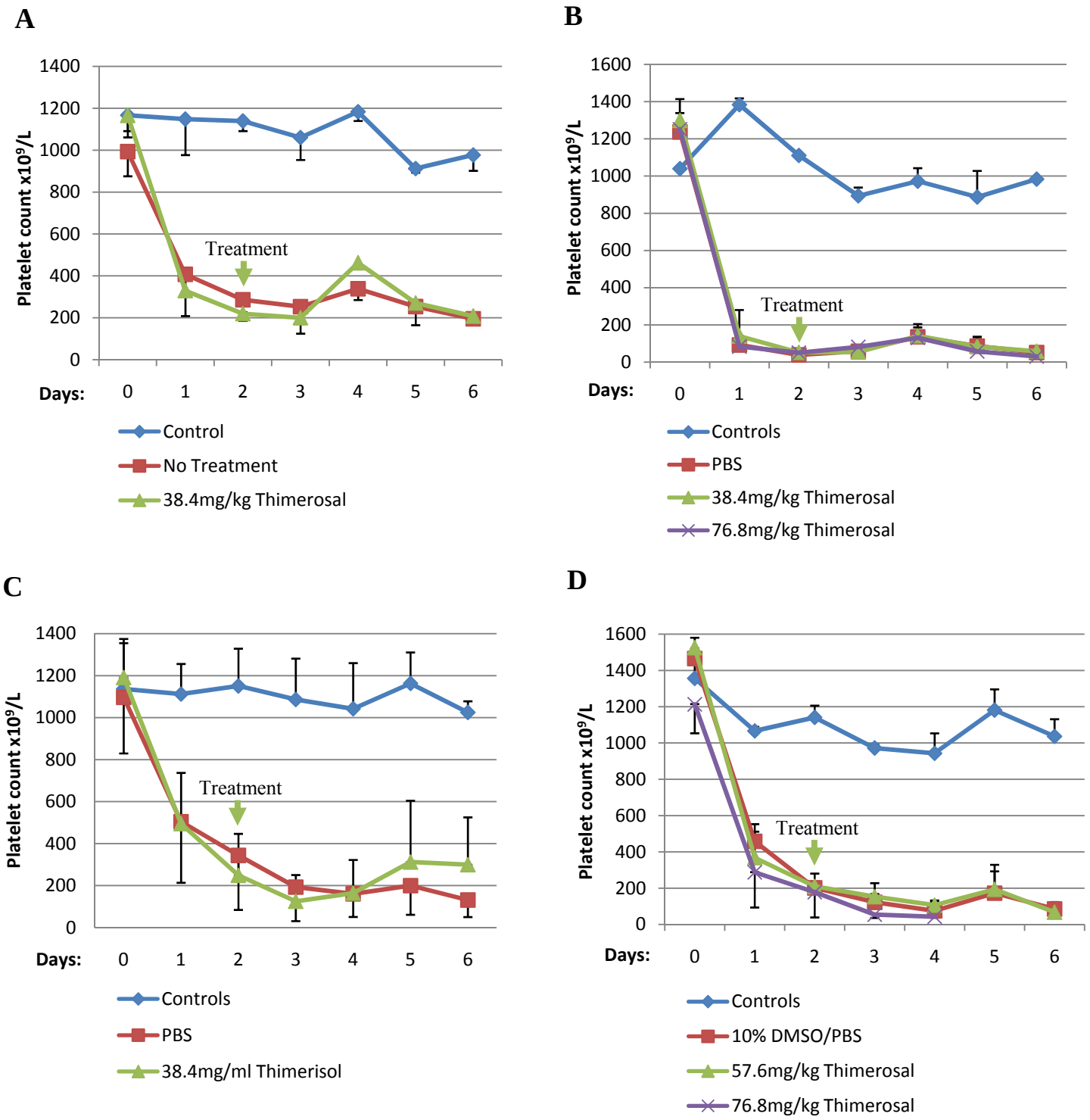


Figure 3.6.1 Efficacy of thimerosal treatment using the TBI-combination and the dose escalation models of PIT:

A. Thimerosal efficacy in CD1 mice using the TBI-combination model of PIT. Mice in the test groups received 4Gy of total body γ-irradiation on day 0, followed by daily administration of 68μg/kg anti-CD41. Mice in the treatment group received 38.4mg/kg thimerosal (in 10% DMSO/PBS) IV on day 2 of the experiment. n=3 mice with bars representing standard deviations. **B**. Thimerosal efficacy in Balb/c mice using the dose-escalation model of PIT (n=4 mice). Mice in all test groups received an escalating dose of anti-CD41 on a daily basis (1.4μg on days 0 and 1, followed by 2.1μg, 2.8μg, 3.5μg and 4.2μg on days 2, 3, 4 and 5, respectively). Control mice received 100μl PBS while test mice received 38.4mg/kg or 76.8mg/kg thimerosal (in PBS), intravenously on day 2 of the experiment. Mice in the group that received 76.8mg/kg thimerosal, died at a rate of one mouse per day, from day 3 of the experiment and onwards. **C**. Thimerosal efficacy in CD1 mice using the dose-escalation model of PIT. Mice in the test groups received an escalating dose of anti-CD41 on a daily basis (2μg on days 0 and 1, followed by 3μg, 4μg, 5μg and 6μg on days 2, 3, 4 and 5, respectively). Control mice received 100μl PBS while test mice received 38.4mg/kg thimerosal (in PBS), intravenously on day 2 of the experiment. n=7 mice with bars representing standard deviations. **D**. Thimerosal efficacy in CD1 mice using the dose-escalation model of PIT. Mice in all test groups received an escalating dose of anti-CD41 on a daily basis (as described in (C)). Control mice received 100μl of 10% DMSO/PBS while test mice received 57.6mg/kg or 76.8mg/kg thimerosal (in 10% DMSO/PBS), intravenously on day 2 of the experiment. All the mice in the group that received 76.8mg/kg thimerosal, died on day 4 of the experiment. n=3 mice with bars representing standard deviations.

3.6.2 Efficacy of first generation compounds using the dose escalation model of PIT

As thimerosal previously was shown to be an effective inhibitor of phagocytosis *in vitro* but showed severe toxicity *in vivo*, it was possible that less toxic structural derivatives would show efficacy in treating PIT *in vivo*. Hence, we started testing chemical compounds that have shown efficacy in phagocytosis inhibition *in vitro* (Rampersad, G.C. *et al.*, 2005), and for which MTDs were previously determined by a contracted company (Nucro-Technics). The MTDs of *p*-nitrophenyl ethanol and *p*-nitrophenyl methyl disulfide (G-B) were determined to be 172.8mg/kg and 51.2mg/kg, respectively (Foo, A.H., 2006). As *p*-toluenesulfonylmethyl mercaptan (F-B) is structurally similar to G-B, we initially tested it at the same MTD (51.2mg/kg). Figure 3.6.2A shows the efficacy of *p*-nitrophenyl ethanol, G-B and F-B when administered IV at 2 and 4 hours post anti-CD41 injection on day 2 of the experiment using the dose escalation model of ITP. The curves shown are average platelet counts with n=6 mice for G-B and F-B, and n=3 mice for *p*-nitrophenyl ethanol. Unfortunately, no significant increase in platelet counts is observed following the administration of these compounds, as compared to control mice that received the diluent alone (10% DMSO/PBS).

Next, we attempted to dissolve G-B, F-B and *p*-nitrophenyl ethanol at as high a concentrations as possible (approximate maximum concentrations of 5mg/ml, 5mg/ml and 17.7mg/ml, respectively). To better solubilise the compounds, they were heated to 60C over night and vigorously mixed by vortexing. No higher concentrations could be tested as the compounds precipitated out of solution, even at these relatively low concentrations. It is difficult to estimate the effective dose of compounds received by the mice, as the majority of the compound formed

chunky sediment at the bottom of the tube. However it is safe to assume that mice received substantially lower doses than calculated in Table 2.5.3.2.

To attempt testing these compounds at higher effective doses, we changed the mode of administration to IP. This allowed us to administer up to four times the volume of a compound as compared to IV administration. The curves obtained with G-B and F-B administered IP at the highest concentration possible (5mg/ml) are shown in Figure 3.6.2B. Unfortunately, the compounds showed no significant effect on platelet counts when administered in this mode.

Figure 3.6.2 Efficacy of first-generation small-molecular-weight compounds using the dose-escalation model of PIT

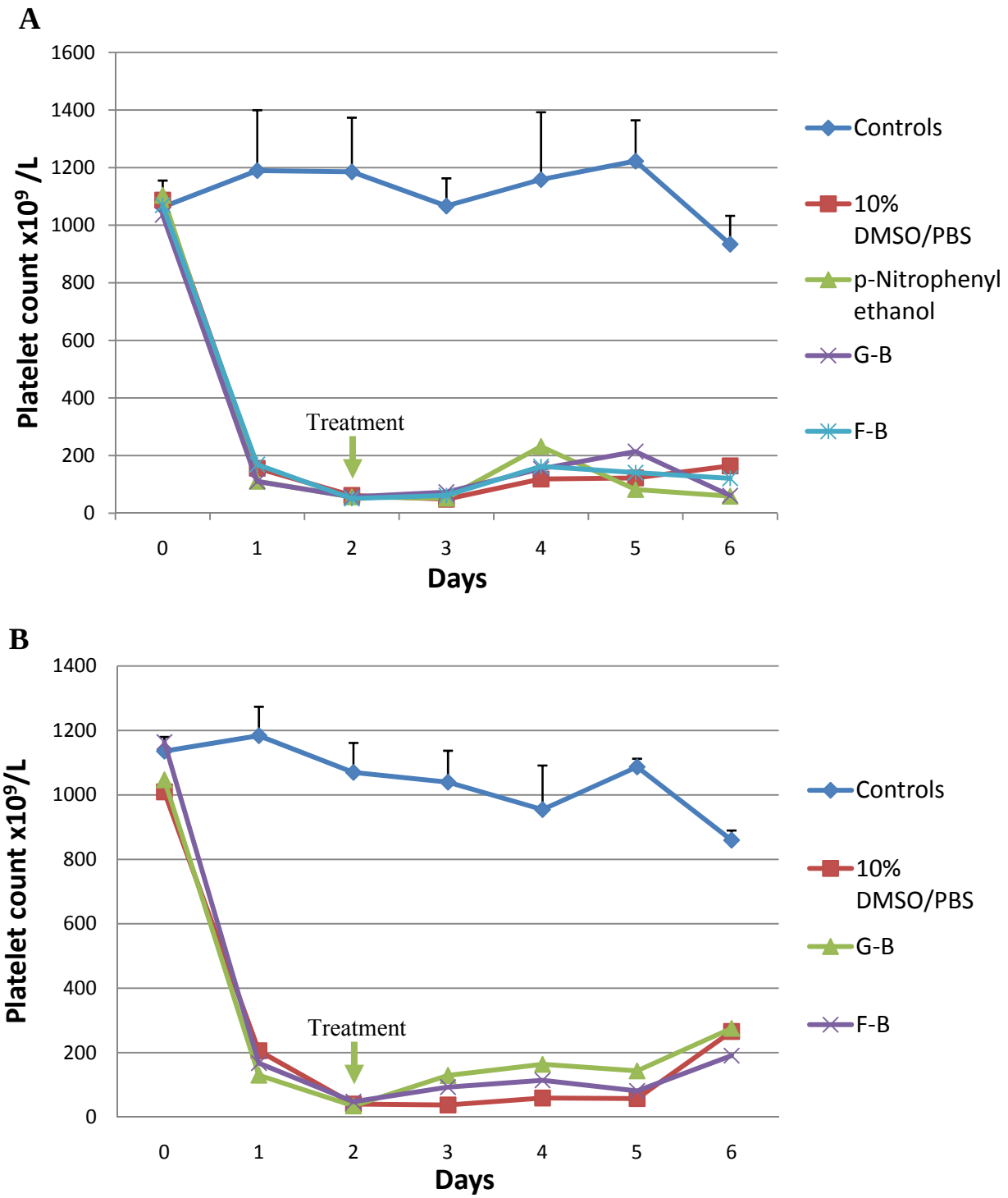


Figure 3.6.2 Efficacy of first generation chemical compounds using the dose escalation mouse model of PIT:

A. Efficacy of first generation compounds when administered IV to Balb/c mice. Mice in all test groups received an escalating dose of anti-CD41 on a daily basis (1.4μg on days 0 and 1, followed by 2.1μg, 2.8μg, 3.5μg and 4.2μg on days 2, 3, 4 and 5, respectively). All treatments were administered in 100μl volumes, intravenously on day 2 of the experiment, 2 and 4 hours post anti-CD41 administration. Control mice received 10% DMSO/PBS (n=9 mice), while test mice received *p*-nitrophenyl ethanol (n=3 mice), *p*-nitrophenyl methyl disulfide (G-B, n=6 mice), or *p*-toluenesulfonylmethyl mercaptan (F-B, n=6 mice) in 10% DMSO/PBS at approximate maximum concentrations of 172.8mg/kg, 50mg/kg or 50mg/kg, respectively (see Table 2.5.3.2 for details and Appendix II.1 for plots of platelet counts of individual mice). **B**. Efficacy of first generation compounds when administered IP to Balb/c mice (n=3 mice). Mice in all test groups received a daily dose of anti-CD41 using the modified dose-escalation protocol (1.4μg on days 0 and 1, followed by 2.1μg on day 2, and 2.8μg every day thereafter). All treatments were administered in 800μl volumes IP on day 2 of the experiment, 2 hours post anti-CD41 administration. Control mice received 10% DMSO/PBS, while test mice received *p*-nitrophenyl methyl disulfide (G-B) or *p*-toluenesulfonylmethyl mercaptan (F-B) in 10% DMSO/PBS at an approximate concentration of 200mg/kg (see Table 2.5.3.2 for details and Appendix II.2 for plots of platelet counts of individual mice).

3.6.3 Efficacy of second generation compounds using the dose escalation model of PIT

As it was apparent that the solubility of a compound was an obstacle for testing its efficacy, we shifted to testing more soluble second generation compounds, including the polar 2,4-dinitrophenyl methyl disulfide (C7), and the sodium salts; *p*-nitrophenyl *ω*-hydrocarboxymethyl disulfide (C10) and *p*-nitrophenyl *ω*-hydrocarboxyethyl disulfide (C11). These newly synthesized compounds did not have official MTDs available. But as they are structural derivatives of G-B, we began by testing them at the G-B MTD, using a step-up step-down approach. Figure 3.6.3 shows the results obtained with the polar C7 and the hydrophilic sodium salts C10 and C11 using the dose escalation model.

The C7 compound formed a milky suspension when dissolved in 10% DMSO/PBS at room temperature. This suspension was uniform at a lower dose (3mg/ml), but reached a saturation point where precipitate was formed at 10mg/ml. Curiously, when heated at 60C for 24 hours or more, the solution became clear, with crystals of the compound coating the tube walls. These crystals increased in size as the concentration of the solution was increased. As it was unclear which form of the compound might show efficacy in elevating platelet counts, both forms were tested by IP administration, in order to maximize the effective dose received by the mice (Fig. 3.6.3A). Unfortunately, no significant effect was observed with either form of this compound.

The sodium salts C10 and C11 did in fact dissolve at a lower concentration (1.28mg/ml), but formed milky suspensions at a higher dose (3mg/ml) in room temperature. The data from two different experiments was combined, and is presented as average platelet counts of n=6 mice (Fig. 3.6.3B). As previously seen with C7, no significant increase in platelet counts was observed following IP administration of C10 or C11 on day 2 of the experiment.

Figure 3.6.3 Efficacy of second-generation small-molecular-weight compounds using the dose-escalation model of PIT

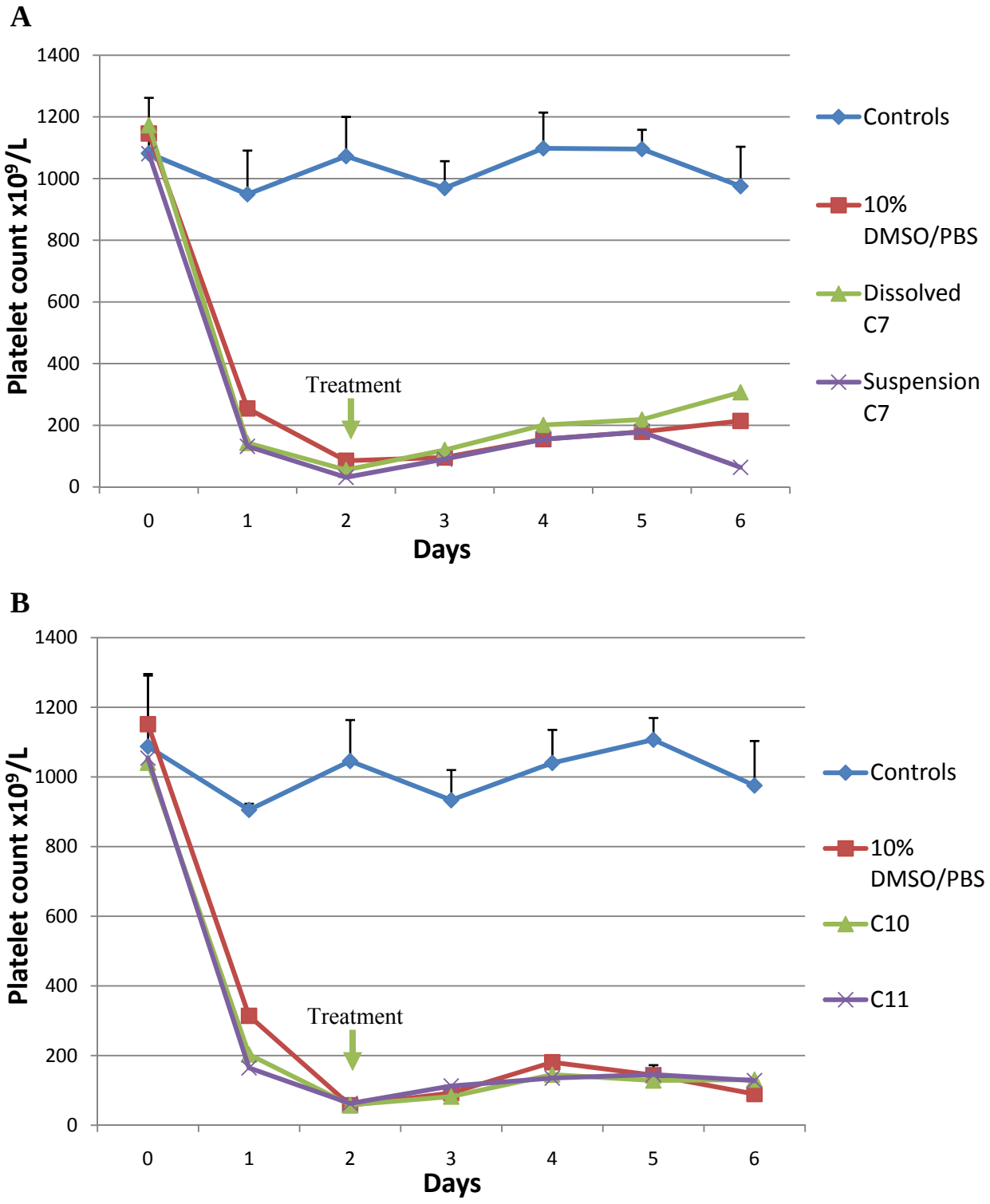


Figure 3.6.3 Efficacy of second generation chemical compounds using the dose escalation mouse model of PIT:

A. Efficacy of 2,4-dinitrophenyl methyl disulfide (C7) in Balb/c mice. Mice in all test groups received a daily dose of anti-CD41 using the modified dose-escalation protocol (1.4μg on days 0 and 1, followed by 2.1μg on day 2, and 2.8μg every day thereafter). All treatments were administered in 800μl volumes IP on day 2 of the experiment, 2 hours post anti-CD41 administration. Control mice received 10% DMSO/PBS (n=9 mice), while test mice received C7 that was either heated at 60C overnight (dissolved, n=6 mice), or that was mixed at room temperature (suspension, n=6 mice). The approximate maximum concentrations of dissolved and suspension C7 administered to mice, are 200mg/kg and 400mg/kg, respectively (see Table 2.5.3.2 for details and Appendix II.3 for plots of platelet counts of individual mice). **B**. Efficacy of second generation sodium salts in Balb/c mice (n=6 mice). Mice in all test groups received a daily dose of anti-CD41 using the modified dose-escalation protocol (as described in (A)). All treatments were administered in 800μl volumes IP on day 2 of the experiment, 2 hours post anti-CD41 administration. Control mice received 10% DMSO/PBS, while test mice received *p*-nitrophenyl *ω*-hydrocarboxymethyl disulfide (C10) or *p*-nitrophenyl *ω*-hydrocarboxyethyl disulfide (C11) at approximate maximum concentrations of 120mg/kg (see Table 2.5.3.2 for details and Appendix II.4 for plots of platelet counts of individual mice).

3.6.4 Toxicity of tested small molecular weight compounds

Thimerosal associated toxicity is described in section 3.6.1.

Considering their limited solubility, we found no significant toxicity associated with *p*-nitrophenyl ethanol, G-B, F-B, C7 and C10 at the tested concentrations. Following the administration of these compounds, the mice appeared healthy, with 100% survival rate. The only compound that had shown associated toxicity was C11. Mice appeared sick and immobile following C11 administration, where increased severity of symptoms correlated with increased dose of C11 administered. The toxicity observed was behavioral in nature, and its underlying physiological basis was not examined.

Chapter 4

Discussion

4.1 Spontaneous platelet recovery is a result of increased thrombopoiesis

While using a current published mouse model of acute immune-mediated thrombocytopenia (Crow, A.R. *et al.*, 2001, Crow, A.R. *et al.*, 2003, Siragam, V. *et al.*, 2005), that utilizes passive same-dose daily administration of anti-platelet antibody, we found ourselves faced with a complication: Platelet counts rose spontaneously in the face of daily anti-platelet antibody administration from day 4 of the experiment and onwards, sometimes reaching normal levels. This 'spontaneous' rebound in platelet counts was observed in our earlier work in CD1 mice (Foo, A.H., 2006), and was confirmed in this work using both CD1 and Balb/c mice (Fig. 3.3.2). It is unclear why this phenomenon was not previously reported in the literature, but one possible explanation is that all earlier reports ended the experiments by day 4, thereby likely overlooking this occurrence (Crow, A.R. *et al.*, 2003, Song, S. *et al.*, 2005).

Interestingly, in recent reports, investigators have shifted to using a modified model of PIT, where IVIG is administered 24 hours prior to the anti-platelet antibody, and platelets are enumerated after another 24 hours (Siragam, V. *et al.*, 2006, Siragam, V. *et al.*, 2005, Leytin, V. *et al.*, 2006). This approach drastically shortens the length of the experiment and shows a protective IVIG effect. Platelet counts of mice in the IVIG pre-treatment group drop only slightly as compared to the marked thrombocytopenia observed in mice that receive the anti-platelet antibody alone (Siragam, V. *et al.*, 2006, Siragam, V. *et al.*, 2005). However, upon testing a similar approach (with a slight modification, as the mice were subjected to 1Gy TBI on day 0),

where IVIG is administered just prior to anti-platelet antibody on day 0, we found that although a protective IVIG effect is evident on day 1, antibody-induced thrombocytopenia is still reached over the course of the experiment, resembling the kinetics seen with anti-platelet antibody alone (Appendix III). The use of a very low dose of 1Gy TBI at day 0 in our experiment likely would not have had any effect on this result, at least in the short term; thus, using this approach, IVIG pre-treatment seems to delay the drop in platelet counts but not prevent it. It is unclear why these investigators chose to use this modified protocol, which does not resemble the human condition (where IVIG treatment is preceded by the development of anti-platelet antibodies), but it is possible that this approach is used to save time and reduce associated costs, due to the anti-platelet antibody being administered only once, as opposed to daily administration. Another likely explanation is that this approach is used to avoid the 'spontaneous' rise in platelet counts which would otherwise interfere with evaluation of IVIG efficacy.

In addition, other investigators have adopted the use of a continuous anti-platelet antibody-delivery system for PIT induction (Teeling, J.L. *et al.*, 2001, Deng, R. & Balthasar, J.P., 2007). Surgically implanted osmotic pumps are used to continuously deliver anti-platelet antibody over the course of the experiment. Although no explanation is given for using this approach, which is more costly and technically difficult to implement as it requires a surgical intervention, it is likely that it is used to prevent spontaneous platelet recovery. This is supported by our personal communications with Dr. Balthasar (University at Buffalo, NY, USA), which suggested that spontaneous platelet recovery was possibly the reason for their use of a continuous anti-platelet antibody-delivery system. Overall, it appears that investigators avoid using the simplest published model of PIT (Crow, A.R. *et al.*, 2001), and employ a range of sometimes more difficult approaches for no explicit reason. However, it is probable that avoidance of the

confounding ‘spontaneous’ increase in platelet counts is the underlying reason for the use of other models.

Unfortunately, as such, the published mouse model of PIT (Crow, A.R. *et al.*, 2001) was unacceptable for our purpose of studying novel treatment modalities for human ITP, as the model was neither able to discern subtle changes in platelet counts nor accurately predict treatment efficacy at later days of the experiment. To address this problem, we first had to identify the underlying mechanism of the observed platelet rebound effect. Our initial hypothesis was that the mouse immune system may mount an immune response against the rat-derived anti-platelet antibodies, resulting in their clearance. This hypothesis was tested using the published model of PIT in CB.17 SCID mice, which are incapable of producing specific antibodies, as they lack T helper lymphocytes and B lymphocytes (Foo, A.H., 2006); thus, these mice cannot mount an immune response producing antibodies to foreign proteins such as the rat immunoglobulin. We have shown that platelet rebound occurs even in these immunodeficient mice (Fig. 3.3.1), suggesting that a mechanism other than immune response resulting in clearance of the anti-platelet antibody was responsible for this phenomenon.

We then decided to test an alternative hypothesis, that the platelet rebound effect was a result of a compensatory bone marrow response resulting in increased thrombopoiesis. It is not unusual for the bone marrow to compensate for a loss of cells in humans, resulting in compensated haemolytic anemia (Fernandez, L.A. & Erslev, A.J., 1972, Erslev, A.J., 1995) or a compensated thrombocytolytic state (Karpatkin, S. *et al.*, 1971, Rodriguez, A.R., 1980). Feedback mechanisms are normally employed by the body to maintain homeostasis. Negative feedback loops, are activated when a change or deviation from a normal range of function is detected, and this change is opposed or resisted to bring the function of the organ or structure back to within the normal range. We hypothesized that such feedback mechanism was responsible for increased

thrombopoiesis resulting in a platelet rebound following a continuous depletion of platelets by the anti-CD41 antibodies. We quantified new thrombopoiesis using thiazole orange-stained reticulated platelets, showing that production of these newly released platelets dramatically increases by day 4-6 of the experiment, mirroring the increase in total platelet counts (Fig. 3.3.2) in both CD1 and Balb/c mice. Curiously the reticulated platelet counts peaked on day 5 and seemed to drop on day 6 despite the continued increase in total platelet counts. This is also likely due to an internal feedback mechanism, whereby the initial increase in platelet counts due to increased thrombopoiesis causes the slowing of thrombopoiesis as platelet counts approach normal levels (Chang, M. *et al.*, 1996); the mouse system realizes that it now has normal platelet numbers, and does not need to produce any more platelets to maintain homeostasis. From these observations we concluded that the platelet rebound was at least partially a result of increased thrombopoiesis in the bone marrow. Our findings suggested that increased thrombopoiesis plays a role in the platelet rebound phenomenon observed in CD1 and Balb/c mice, and likely in CB.17 SCID (Fig. 3.3.1, but reticulated platelets not examined), suggesting this rebound effect may be a universal phenomena.

Notably, the increase in reticulated platelet counts, although clearly significant, could not account for the large increases in total platelet counts. Although thrombopoiesis clearly plays an important role in the observed platelet rebound, other factors could potentially contribute to the rise in total platelet counts as well. One possibility is the release of sequestered platelets that are stored within the spleen (Penny, R. *et al.*, 1966), in response to continually low total platelet counts. Another possible explanation is that young platelets are not cleared by the MPS as efficiently as older platelets, due to a differential distribution of the anti-platelet antibody on the surface of young versus older platelets. As reticulated platelets are larger than mature platelets (Stenberg, P.E. & Levin, J., 1989), the distribution of receptors on their cell surface may not

favour recognition and/or clearance by the MPS. Finally, enhanced clearance of the anti-platelet antibody itself may contribute to the platelet rebound effect. As the body is exposed to daily doses of the anti-CD41 antibody, it may begin to recognize and respond to its presence in a faster and more efficient manner, resulting in increased antibody clearance at later days of the experiment. However, although some or all of these possible explanations may in fact account for the discrepancy between the increase in reticulated and total platelet counts, we hypothesized that the platelet rebound could be circumvented by minimizing thrombopoiesis in the bone marrow.

4.2 Sub-lethal total body irradiation in combination with passive daily anti-platelet antibody administration is effective at preventing a spontaneous platelet rebound

As increased thrombopoiesis was at least in part the cause of platelet recovery using the previously reported mouse model (Crow, A.R. *et al.*, 2003), we hypothesized that sub-lethal doses of TBI would sufficiently suppress the bone marrow and thrombopoiesis (Gallicchio, V.S., 1988), thereby preventing the platelet rebound effect. TBI alone affected the platelet counts in a dose-dependent manner; the higher the dose of TBI, the greater the suppression of platelet thrombopoiesis from day 6 post TBI and onwards (Fig. 3.4.1A). This finding is consistent with the life span of mouse platelets being 4.5 – 5.7 days, depending on the strain (Ault, K.A. & Knowles, C., 1995, Manning, K.L. & McDonald, T.P., 1997) – as older platelets die, and young platelets are not generated by the TBI-suppressed bone marrow, total platelet counts begin to drop**.** When sub-lethal doses of TBI were combined with same dose passive daily anti-platelet antibody administration, the thrombocytopenic state of mice was maintained throughout the length of the experiment (Fig. 3.4.1B). The underlining mechanism of thrombopoiesis suppression was clearly demonstrated by the reticulated platelet counts (Fig. 3.4.1C), which remained at background levels and failed to increase over the course of the experiment.

One interesting observation is that slight increases in platelet counts were always observed on days 4-5 with this model, effectively resulting in a ‘bump’ on the curve as opposed to having a flat line. Unfortunately, the reason for this phenomenon is unclear. It may be related to a lack of total bone marrow suppression due to the use of sub-lethal TBI, combined with a strong feedback loop mechanism (Chang, M. *et al.*, 1996) in the animals to produce additional platelets. However, after day 5 the bone marrow suppression is more pronounced preventing any production of platelets due to a feedback mechanism. This notion is supported by the fact that

this model works similarly with TBI doses as low as 1Gy (Appendix III), with the size of a 'bump' in the curve inversely proportional to the dose of TBI. Other possible explanations for the presence of this 'bump' are production of thrombopoietic cytokines (such as TPO, IL-6 and IL-11), or induction of radio-protective cytokines (such as IL-1, TNF-α, and stem cell factor (SCF)). Thrombopoietic cytokines could be produced as a result of a feedback mechanism, while radio-protective cytokine-production could be induced by the γ-irradiation. The radio-protective cytokines may exert their effects by induction of cycling of primitive progenitor cells (IL-1, SCF), prevention of apoptosis (SCF), and induction of scavenging proteins and enzymes (IL-1, TNF-α) that reduce oxidative damage (Neta, R., 1997). The finding that higher radiation doses are associated with weaker efficacy of haematopoietic growth factors (Herodin, F. & Drouet, M., 2005) is consistent with the size of a 'bump' being inversely proportional to the dose of TBI.

We have also demonstrated the necessity of daily anti-platelet antibody administration for continued maintenance of severe thrombocytopenia. TBI alone was not sufficient to prevent platelet rebound and maintain low platelet counts, keeping these close to nadir, again likely due to a feedback mechanism (Fig. 3.4.2). When the antibody is given only once, the bone marrow suppression is not yet strong enough to withstand counteraction by a strong feedback mechanism, which results in platelet counts gradually reaching normal, and even higher than normal levels. However, when a single dose of anti-platelet antibody is missed at a later stage of the experiment, the rebound is slight, but noticeable. This demonstrates the powerful additive effect achieved by a combination of daily anti-platelet antibody administration and TBI, and suggests that the bone marrow suppression achieved by sub-lethal doses of TBI alone is insufficient for complete suppression of thrombopoiesis.

The TBI-antibody combination mouse model appears to be an improved model of immune thrombocytopenia, because in order to be able to test potential alternative treatment modalities (that could have subtle effects in reversing immune-mediated platelet destruction), maintenance of the thrombocytopenic state is imperative. However, treatment modalities to be examined must improve the platelet counts significantly or the animal model is of no benefit. Therefore, we tested the efficacy of IVIG using the new TBI-antibody combination model. As the mechanism of IVIG effect in the mouse model of PIT have been reported to involve up-regulation of the inhibitory FcγRIIB receptor (Samuelsson, A. *et al.*, 2001, Siragam, V. *et al.*, 2006), we were surprised to find out that IVIG was ineffective in elevating platelet counts, particularly at the higher TBI doses. The efficacy of IVIG, although insignificant at the TBI levels we tested, was nonetheless inversely proportional to the dose of TBI. The lack of efficacy of IVIG can thus be attributed to the γ-irradiation effects and provides some information regarding the possible mechanism of IVIG effect to increase platelet counts in the mouse PIT model. As TBI is not specifically targeted to affect the bone marrow, but instead affects the whole animal, it may affect a variety of cells, tissues and organs. It may impair the function of megakaryocytes, resulting in impaired thrombopoiesis, or induce apoptosis of cells. Alternatively, TBI may affect some radio-sensitive regulatory cells, such as dendritic cells (Siragam, V. *et al.*, 2006), possibly disrupting a cytokine network and/or interrupting downstream signalling cascades which are important for the IVIG effect. Alternatively, our results may indicate that the mechanism of IVIG does not involve dendritic cells or the regulatory FcγRIIB receptor but requires a completely functional bone marrow, and that IVIG has its effect on thrombopoiesis stimulation. However, a radio-sensitive effector cell may induce a pathway (for example, by cytokine production) that can interact with and stimulate production of platelets in the bone marrow. Determination of the mechanism of action of IVIG is beyond the scope of this thesis. Regardless

of the mechanism, our results may have implications for cancer patients who receive radiation and/or cytotoxic drug therapy, and imply that IVIG may be less effective or ineffective treatment for ITP amelioration in these patients.

Anti-TER-119 is a monoclonal anti-erythrocyte–specific (Kina, T. *et al.*, 2000) antibody that is used to mimic the therapeutic effects of anti-D in ITP (Song, S. *et al.*, 2003). We have tested its efficacy in the TBI/anti-platelet antibody combination model as it has been shown to ameliorate murine ITP by a mechanism different than that of IVIG (Song, S. *et al.*, 2005). Our results support this finding, as anti-TER-119, unlike IVIG, was effective at significantly increasing platelet counts even when mice were subjected to a higher dose of TBI (4Gy). The TBI combination model clearly allows a differentiation between the general targets underlying the mechanisms of action of various treatments. Consequentially, the efficacy of therapeutic agents that affect the FcγR or the clearance of antibody, as opposed to agents that directly or indirectly regulate thrombopoiesis, could be reliably explored using the TBI combination model.

4.3 Gradual dose escalation of the anti-platelet antibody successfully maintains the antibody-induced thrombocytopenia

We required a mouse model that would maintain antibody-induced thrombocytopenia over time but also respond to IVIG treatment. Therefore we attempted to develop an alternative model that would eliminate the undesirable side effects of TBI. Initially, we showed that the proportion of anti-CD41 coated cells does not change over time, by examining the proportion of antibody-coated platelets over the course of the experiment (Fig. 3.3.3). This suggested that anti-CD41 is able to bind reticulated platelets as well as mature platelets, as previously shown (Debili, N. *et al.*, 2001, Panasiuk, A. *et al.*, 2004). In addition, we have found that the high availability (close to 100%) of epitopes for anti-CD41 binding *in vivo*, does not change over the course of the experiment, suggesting that anti-platelet antibodies never saturate the platelets at the dose we administer (68μg/kg). These findings together with the idea that anti-platelet antibodies would have a finite half-life in circulation led us to hypothesize that by increasing the levels of anti-CD41 we would be able to prevent the platelet rebound effect. That is because increasing levels of anti-platelet antibody would potentially compensate for the increased reticulated platelet production, which likely results from a feedback loop combating the low platelet counts induced in PIT. This approach is similar to that used by Teeling, J.L. *et al.* (2001) and Deng, R. & Balthasar, J.P. (2007) where implanted osmotic pumps are used to continuously deliver the anti-platelet antibody to the blood stream of mice, thereby effectively maintaining the thrombocytopenic state in mice over longer periods of time. As the surgical approach is expensive and technically difficult to implement, we thought that a similar thrombocytopenia could be achieved by gradually increasing over time the single daily dose of anti-CD41 given IP. Employing this dose-escalation model, we demonstrated that both CD1 and Balb/c mice

maintained low platelet counts throughout the course of the experiment. We believe that the dose-escalation model strikes a balance between generation of new platelets by thrombopoiesis and platelet clearance by phagocytosis, by increasing the amount of antibody coated platelets over time. This is evident from the reticulated platelet counts, which remain at background levels over the course of the dose-escalation (Fig. 3.5.1B and 3.5.2C).

When we tested the effectiveness of IVIG in elevating platelet counts in the dose-escalation model, we found that, unlike with the TBI model, the IVIG effect was clearly significant. Although platelet counts did not reach the normal range, as reported with IVIG treatment (Crow, A.R. *et al.*, 2003, Blanchette, V. & Carcao, M., 1998), the increase in platelet counts was nonetheless highly significant, providing us with a sensitive and effective new model for the study of immune thrombocytopenia. The lack of a complete therapeutic effect (reaching normal platelet numbers) may be due to the high levels of anti-CD41 (>170μg/kg after day 4) affecting megakaryocyte viability and/or function. Indeed, we were able to show an increased efficacy approaching normal platelet numbers following IVIG administration using a slightly modified dose-escalation protocol as shown in Figure 3.5.3, where the escalation was held at 136μg/kg from day 3 onwards. Curiously, in all our experiments, IVIG efficacy was transient in nature, with platelet counts dropping on day 6 after reaching a peak on day 5. This finding suggests that the IVIG effect may be wearing off, possibly due to its clearance from the system or desensitization to its effects. A similar phenomenon is documented in humans, with patients requiring maintenance doses of IVIG to maintain their remission (Gelfand, E.W., 2006, Fehr, J. *et al.*, 1982). In summary, the dose escalation model is successful in maintaining the antibody-induced thrombocytopenia and most closely resembles the human condition of ITP. As such, and lacking the confounding effects of γ-irradiation, it was chosen for testing of our candidate compounds for PIT amelioration.

4.4 Efficacy of small-molecular-weight compounds *in vivo*

Thimerosal is a sulfhydryl-reactive potent inhibitor of phagocytosis *in vitro* (Rampersad, G.C. *et al.*, 2005). However due to the presence of a mercury atom in its structure, it is quite toxic at high concentrations, both *in vitro* and *in vivo* (Rampersad, G.C. *et al.*, 2005, Foo, A.H., 2006, Branch, D.R., 2009). It is difficult to assess thimerosal's efficacy in PIT amilioration *in vivo*, as its toxic effects are evident even at low concentrations (38.4mg/kg and 57.6mg/kg), and become profound, resulting in death at the highest concentration tested (76.8mg/kg). It is unclear as to why the MTD we are using appears to be inaccurate based on the contracted results obtained from Nucro-Technics (Foo, A.H., 2006). However, we were unable to demonstrate thimerosal efficacy in PIT amelioration, likely due to its toxicity.

Curiously, it appears as though thimerosal may have better efficacy when the TBI-combination model is used, as compared to the dose escalation model. Although these findings may potentially be a result of human error, it is also possible that efficacy of thimerosal is in-fact more discernible using the TBI-combination model. We chose to use the dose escalation model for our studies, because IVIG efficacy was detectable with this model. However, IVIG likely works by a completely different mechanism than our small-molecular-weight candidate compounds. Evidence in our laboratory, including the finding that reticulated platelet counts mirror the increase in total platelet counts following IVIG administration (Fig. 3.5.2), suggest that IVIG works at least in part, by stimulation of thrombopoiesis. This mechanism of IVIG action also explains its lack of efficacy in the TBI-combination model. As the bone marrow is suppressed by γ-irradiation, IVIG is unable to stimulate thrombopoiesis. In contrast, our small-molecular-weight candidates are designed to inhibit FcγR-mediated phagocytosis. As such, they are not expected to have an effect on thrombopoiesis, making the suppressed bone marrow in the

TBI-combination model irrelevant. This could possibly be a more sensitive model for detection of compound efficacy. However, we have chosen to use the dose-escalation model for further testing of our candidate compounds, as it more closely resembles the human condition.

Although thimerosal has shown no efficacy in elevating platelet counts in our dose escalation model of PIT, we tested less toxic sulfur-reactive chemical compounds in hope we could detect efficacy in the absence of toxicity. As these newly-synthesized chemical compounds contain SH or SS groups, they were expected to interact with either SS linkages on FcγRs (Sondermann, P. *et al.*, 2001), or with adjacent SH group-containing proteins, respectively, thereby inhibiting the interaction of antibody Fc with the FcγRs. Unfortunately, the less toxic, first generation newly synthesized compounds *p*-nitrophenyl ethanol, *p*-nitrophenyl methyl disulfide and *p*-toluenesulfonylmethyl mercaptan, posed a new challenge. Due to their hydrophobic nature, we could not effectively dissolve these compounds in an aqueous solution (PBS) at a high enough concentration that could potentially be effective in ITP amelioration. The compounds precipitated out of solution even when 10% DMSO, which is a polar aprotic solvent capable of dissolving non-polar compounds and miscible in water, was used. As a result, these compounds were tested at sub-optimal concentrations, and were found to be generally ineffective. Also, the administered compounds may have been depleted prior to their interaction with phagocytes as a result of non-specific interactions with proteins on the surface of other cell populations via their sulfhydryl and disulfide groups. To address this complication, very high concentrations of compounds may have to be administered for sufficient number of compound molecules to reach and directly interact with FcγRs on macrophage surfaces. The possibility that if administered at higher enough doses, these compounds may exhibit efficacy *in vivo*, remains to be tested. However, that would require a change in the mode of administration to accommodate the physical properties of our compounds.

It is unclear whether the mode of administration (IV or IP) plays a role in discerning compound efficacy. Using IV administration we were unable to test high enough doses of candidate compounds, resulting in no perceived efficacy. Alternatively, IP administration allowed us to deliver up to four times the dose of a test compound. However, we found no significant efficacy delivering higher doses of test compounds IP, possibly because the compounds may have not readily reached the blood stream, where they are expected to exert their inhibitory effect on phagocytosis.

To minimize the challenge posed by the solubility of our first generation compounds, we tested second generation compounds that were designed to have increased solubility while maintaining the desirable functional groups (a phenyl ring with disulfide and nitro side chains). Unfortunately, these compounds (2,4-dinitrophenyl methyl disulfide, *p*-nitrophenyl ω-hydrocarboxymethyl disulfide and *p*-nitrophenyl ω-hydrocarboxyethyl disulfide) showed no efficacy when administered IP using the dose escalation model of PIT. Both the mode of administration as well as a maximum solubility limit could be responsible for the lack of efficacy of these compounds. Our compounds reached a solubility limit, despite their more soluble chemical properties (C7 being a polar compound and C10 and C11 being sodium salts). As such, although we were able to test these compounds in higher concentrations than our first generation compounds, the concentrations were likely not high enough to discern efficacy. As well, it is possible that the IP mode of administration reflected negatively on compound efficacy, if the compounds did not readily reach the bloodstream. Also, as ITP is a disease where the detrimental effect of auto-antibody production is compounded by impaired thrombopoiesis, it is possible that phagocytosis inhibition alone, even if achieved by our compounds, may not be sufficient for PIT amelioration.

Most of our newly synthesized compounds showed no toxicity at the tested concentrations. The only exception was the C11 compound, which proved to be somewhat toxic to the mice. As C11 differs from C10 only in the length of the ionic group-carrying disulfide substituent (with C11 containing an extra CH_2 group), the toxicity of C11 must be a result of this difference. It is possible that the longer side chain allows the molecules of C11 to better interact with one another forming the perceivable needle like crystals (Table 2.5.3.2) within the peritoneal cavity. These crystals likely have difficulty entering the bloodstream, and may exert toxicity by damaging internal organs. Alternatively, the increased length of the side-chain containing the disulfide substituent may make this moiety more available for interaction with proteins, possibly causing toxicity through interaction with critical proteins.

Chapter 5

Summary and Future Directions

In summary, in this work we describe two new mouse models for the study of passive antibody-induced immune thrombocytopenia where antibody-induced thrombocytopenia is maintained close to nadir throughout the course of the experiment. The TBI/anti-platelet antibody combination model may be useful for investigations of treatment modalities that are thought to target effector cells, such as FcγR blockers, or mechanisms that affect the clearance of antibody. This mouse model should also be useful for investigating potentially effective therapeutics for reversing immune thrombocytopenia in cancer patients that may be refractory to IVIG due to bone marrow suppression. In addition, the dose-escalation mouse model shown in Figure 3.5.3 is currently the best available protocol for investigations of treatment modalities given on day 2 following induction of immune thrombocytopenia, and should allow for the reliable study of various anti-thrombocytopenic agents. However, it appears that megakaryocyte function may be adversely affected by higher doses of anti-platelet antibody, possibly being a confounding factor in discerning therapeutic efficacy. To test whether megakaryocytes are in fact affected, a bone marrow analysis could be performed on days 3-6 of the experiment under different anti-CD41 dose-escalation approaches. If the megakaryocytes were found to be adversely affected by the high doses of anti-CD41 antibody, the dose of antibody given from day 2 onwards could be varied and further refined, in an effort to determine the most optimal dose-escalation approach that would still maintain continuous thrombocytopenia keeping it close to the nadir.

Unfortunately, none of our sulfur-containing candidate chemical compounds tested have shown efficacy in elevating platelet counts using the dose-escalation model of PIT. This may be due to the chemicals physical properties that limit their solubility in aqueous solutions. To be able to test the real potential of these compounds, a more optimal delivery system must be used. One solution is to administer these compounds orally, in order for them to reach the blood stream via intestinal absorption. This approach is commonly used for hydrophobic drugs (Gursoy, R.N. & Benita, S., 2004), but it must be optimized for our compounds to ensure consistent delivery and absorption.

As our compounds are delivered IV or IP, a difficulty we are faced with is specific targeting. As sulphur-reactive compounds can interact with a range of proteins, the compounds administered IV or IP may be depleted before reaching FcγRs on macrophage surfaces. Thus, high concentrations of compounds must be administered to increase the likelihood of our compounds directly interacting with macrophage surfaces. One way to overcome this problem is by liposome-loaded delivery (Cao, Y. & Suresh, M. R., 2000), specifically targeted to macrophage surfaces by macrophage-specific antibodies. However, although liposome-loaded delivery will overcome the challenge posed by the hydrophobicity of our compounds, it will only allow for delivery of compounds by endocytosis. As this would occur primarily by macrophages we would have appropriate targeting of our compounds; however, as the compounds would be released inside the cells, a potential problem would arise because the compounds are designed to target FcγRs on the cell surface.

An alternative approach is to optimize the chemical structures of our compounds to be more water soluble. As the tested carboxylic acid salts (C10 and C11) were not soluble enough, these compounds could be synthesized and tested as sulfonic acid or trimethyl ammonium salts. Either type of structure will likely provide increased water solubility. We are planning on consulting with Dr. Lakshmi Kotra, director of the Molecular Design and Information Technology Center in University Health Network (Toronto, ON), regarding further drug optimization and design. Once we are able to come up with optimal molecular design, new compounds could be synthesized by Dr. Adrian Schwan, our collaborator from the Department of Chemistry at the University of Guelph (Guelph, ON).

Chapter 6

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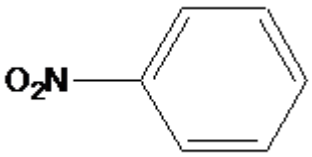
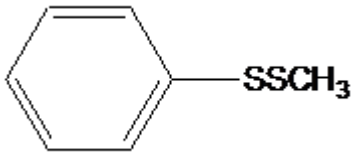
Appendix I – Structures of Additional Newly-Synthesized Chemical Compounds

A



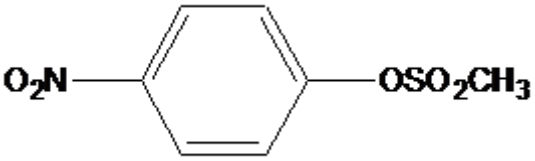
nitrobenzene

B



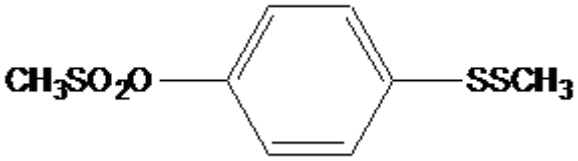
phenyl methyl disulfide

C



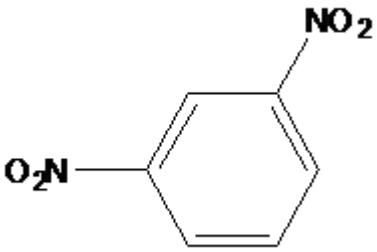
p-nitrophenyl methanesulfonate

D



p-methylsulfonoxyphenyl methyl disulfide

E

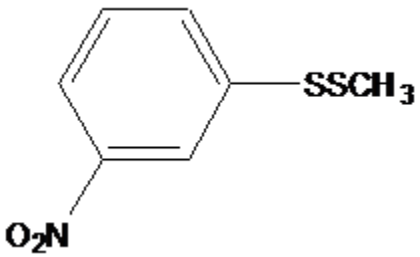


m-dinitrobenzene

F

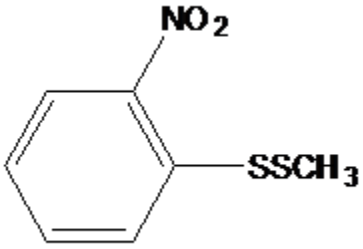
p-di(dithiapropyl)benzene

G



m-nitrophenyl methyl disulfide

H



o-nitrophenyl methyl disulfide

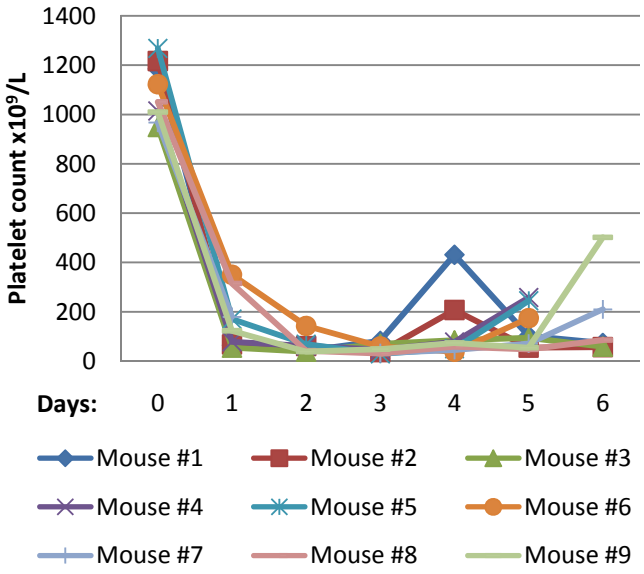
Appendix I Structures of chemical compounds synthesized by Dr. Richard Langer but not tested in this thesis due to their limited solubility in aqueous solutions. These chemical structures are of second generation compounds, designed based on the structure of *p*-nitrophenyl methyl disulfide (G-B, Fig. 2.5.3B). **A. and B.** Nitrobenzene (C1) and phenyl methyl disulfide (C2) were designed to examine the individual roles played by each of the functional groups of G-B separately. As such, nitrobenzene only has a nitro group, while phenyl methyl disulfide has only a disulfide functional side chain. **C. and D.** *p*-nitrophenyl methanesulfonate (C3) and *p*-methylsulfonoxyphenyl methyl disulfide (C4) were designed to increase water solubility of C1 and C2 by adding a polar methanesulfonate group to each compound. **E. and F.** *m*-dinitrobenzene (C5) and *p*-di(dithiapropyl)benzene (C6) were designed to test the effect of having two identical functional groups in a given molecule, in case either functional group (nitro or disulfide) proved to be essential for compound efficacy. **G. and H.** *m*-nitrophenyl methyl disulfide (C8) and *o*-nitrophenyl methyl disulfide (C9) were designed to test whether the position of the functional groups in relation to one another in G-B has an effect on the efficacy of the compound. As such, the functional groups are placed meta- and ortho-, in C8 and C9, respectively, as opposed to para- in G-B.

NOTE. An additional sulfhydryl compound ($PhC(O)CH_2SH$) was synthesized based on the structure of *p*-toluenesulfonylmethyl mercaptan (F-B, Fig. 2.5.3D) (Andrews, C.G. *et al.*, 2009). This *p*-acyl mercaptan, properly named benzoylmethyl mercaptan (C13), could have been tested for its efficacy. However, its limited water solubility posed a challenge for effective administration to mice, as was the case with all the compounds listed above.

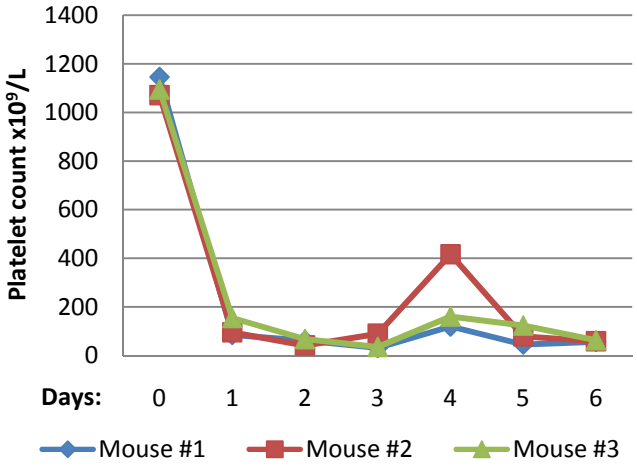
Appendix II – Response of individual mice to the tested small-molecular-weight sulfur compounds under various conditions (by experiment)

Appendix II.1 – Efficacy of first-generation small-molecular-weight compounds when administered IV to Balb/c mice

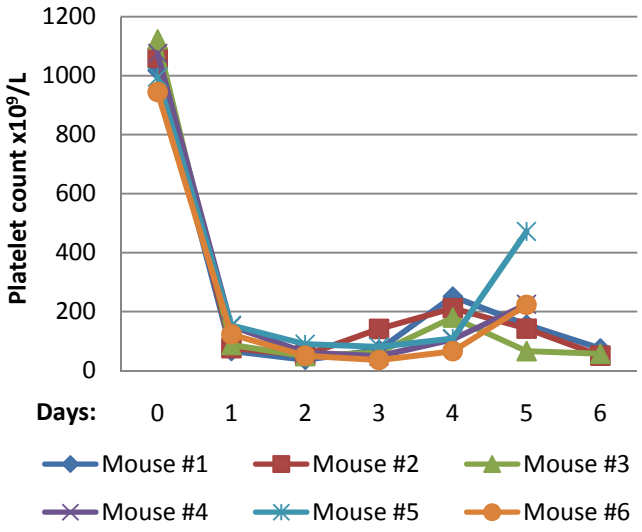
A



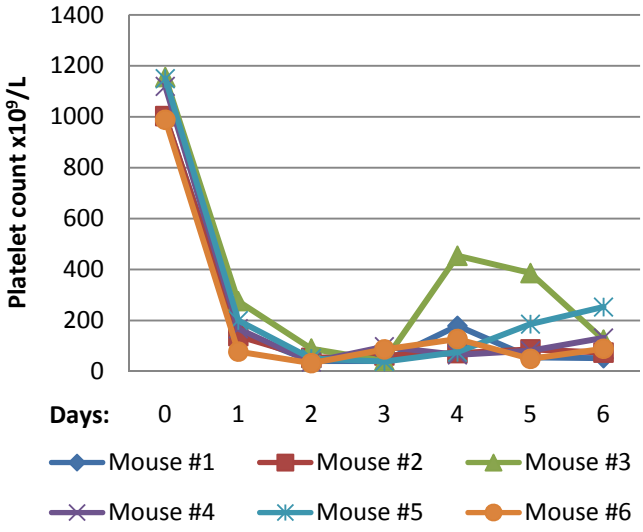
B



C



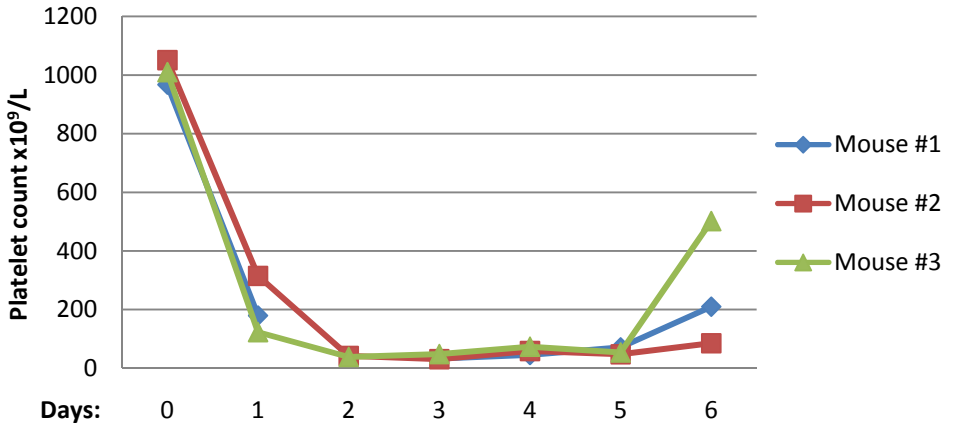
D



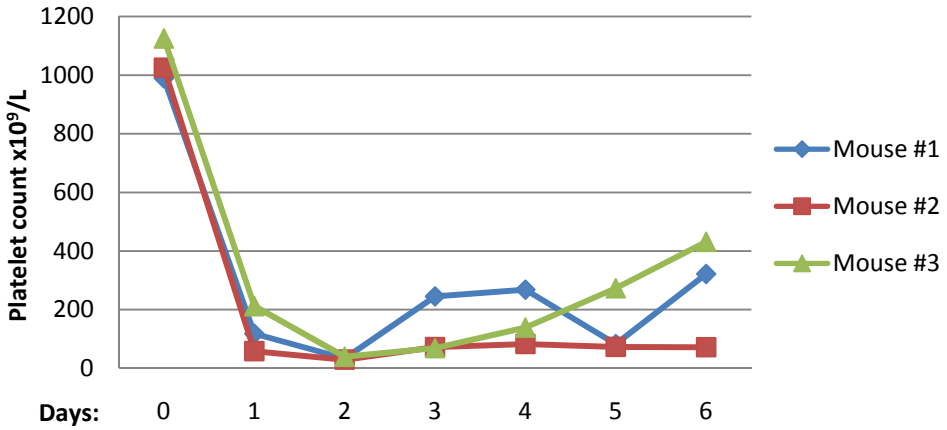
Appendix II.1 Efficacy of first generation chemical compounds using the dose escalation mouse model of PIT in Balb/c mice. All compounds were administered in 100µl volumes, intravenously on day 2 of the experiment, 2 and 4 hours post anti-CD41 administration. Data from different experiments was pooled together, resulting in variability in sample sizes between different groups. Platelet counts of individual mice in each group are shown. **A**. 10% DMSO/PBS. **B**. *p*-nitrophenyl ethanol in 10% DMSO/PBS (approximate maximum concentration = 172.8mg/kg). **C**. *p*- nitrophenyl methyl disulfide (G-B) in 10% DMSO/PBS (approximate maximum concentration = 50mg/kg). **D**. *p*-toluenesulfonylmethyl mercaptan (F-B) in 10% DMSO/PBS (approximate maximum concentration = 50mg/kg). This data constitutes the mean platelet counts shown in Figure 3.6.2A. Please refer to Table 2.5.3.2 for detailed information on the preparation of compounds.

Appendix II.2 – Efficacy of first-generation small-molecular-weight compounds when administered IP to Balb/c mice

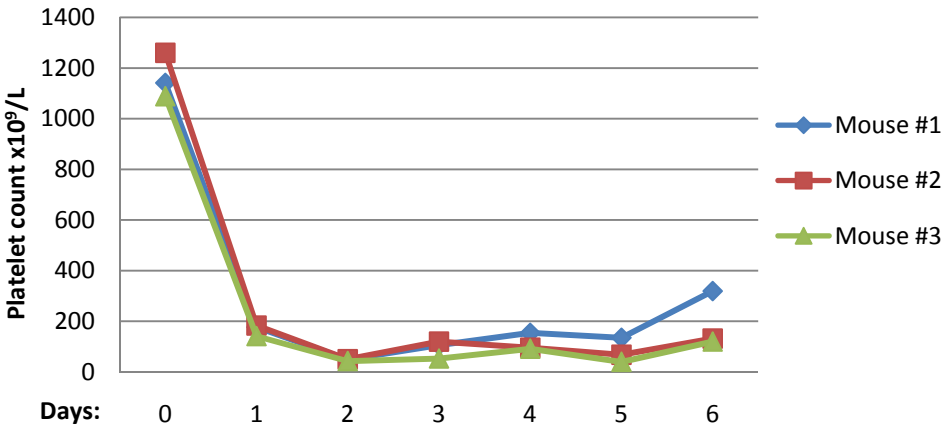
A



B



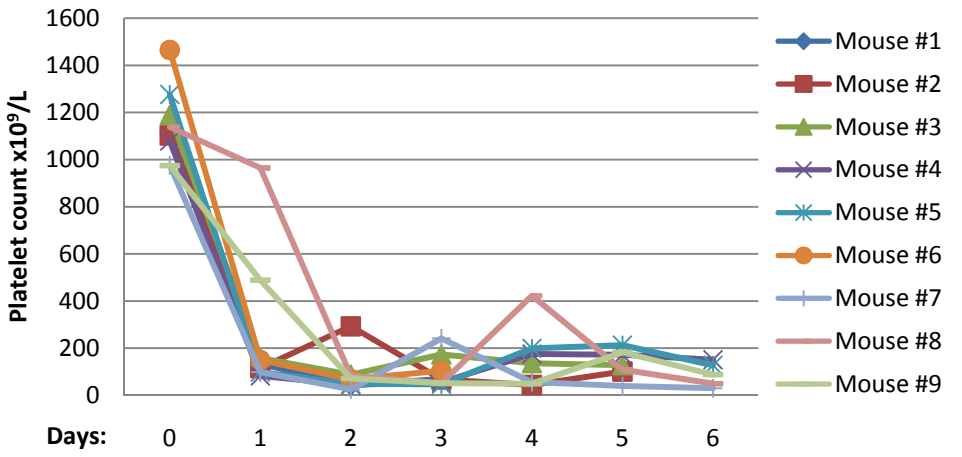
C



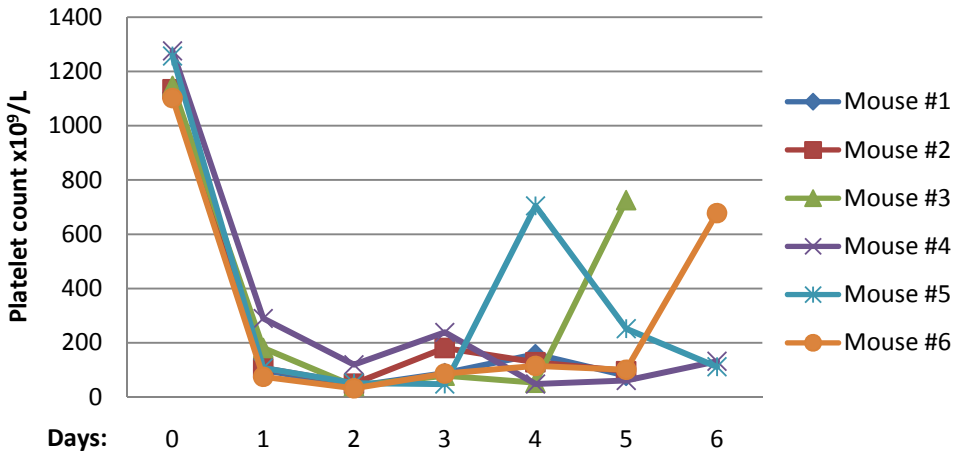
Appendix II.2 Efficacy of first generation chemical compounds using the dose escalation mouse model of PIT in Balb/c mice. All compounds were administered in 800µl volumes IP on day 2 of the experiment, 2 hours post anti-CD41 administration. Platelet counts of individual mice in each group are shown. **A**. 10% DMSO/PBS. **B**. *p*-nitrophenyl methyl disulfide (G-B) in 10% DMSO/PBS (approximate maximum concentration = 200mg/kg). **C**. *p*-toluenesulfonylmethyl mercaptan (F-B) in 10% DMSO/PBS (approximate maximum concentration = 200mg/kg). This data constitutes the mean platelet counts shown in Figure 3.6.2B. Please refer to Table 2.5.3.2 for detailed information on the preparation of compounds.

Appendix II.3 – Efficacy of 2,4-dinitrophenyl methyl disulfide (C7) when administered IP to Balb/c mice

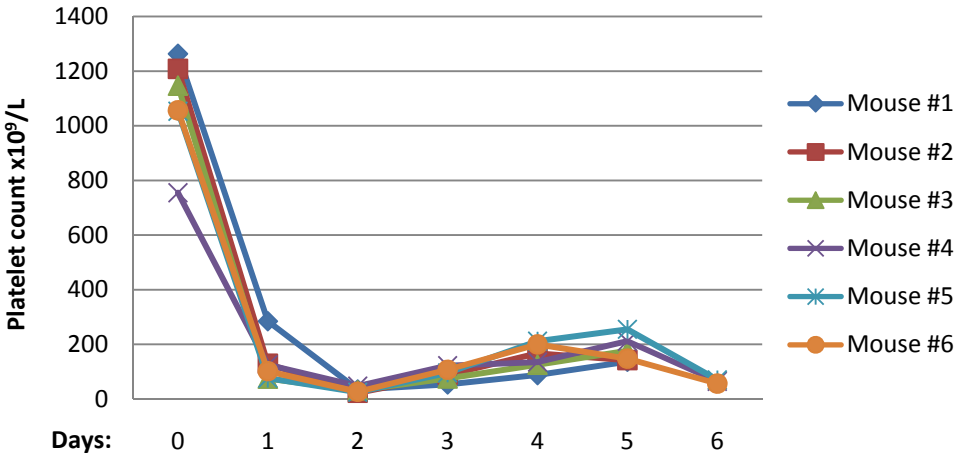
A



B

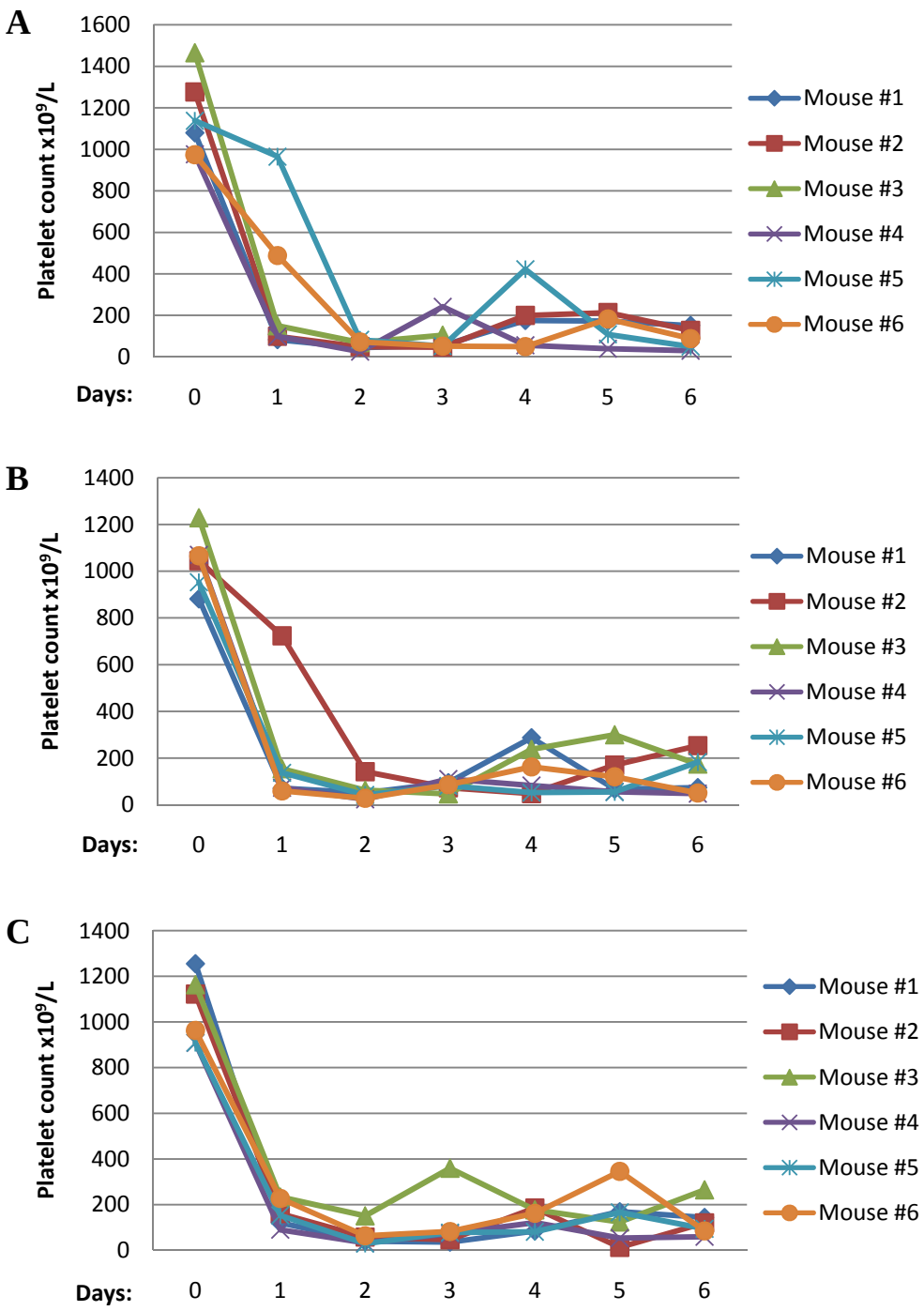


C



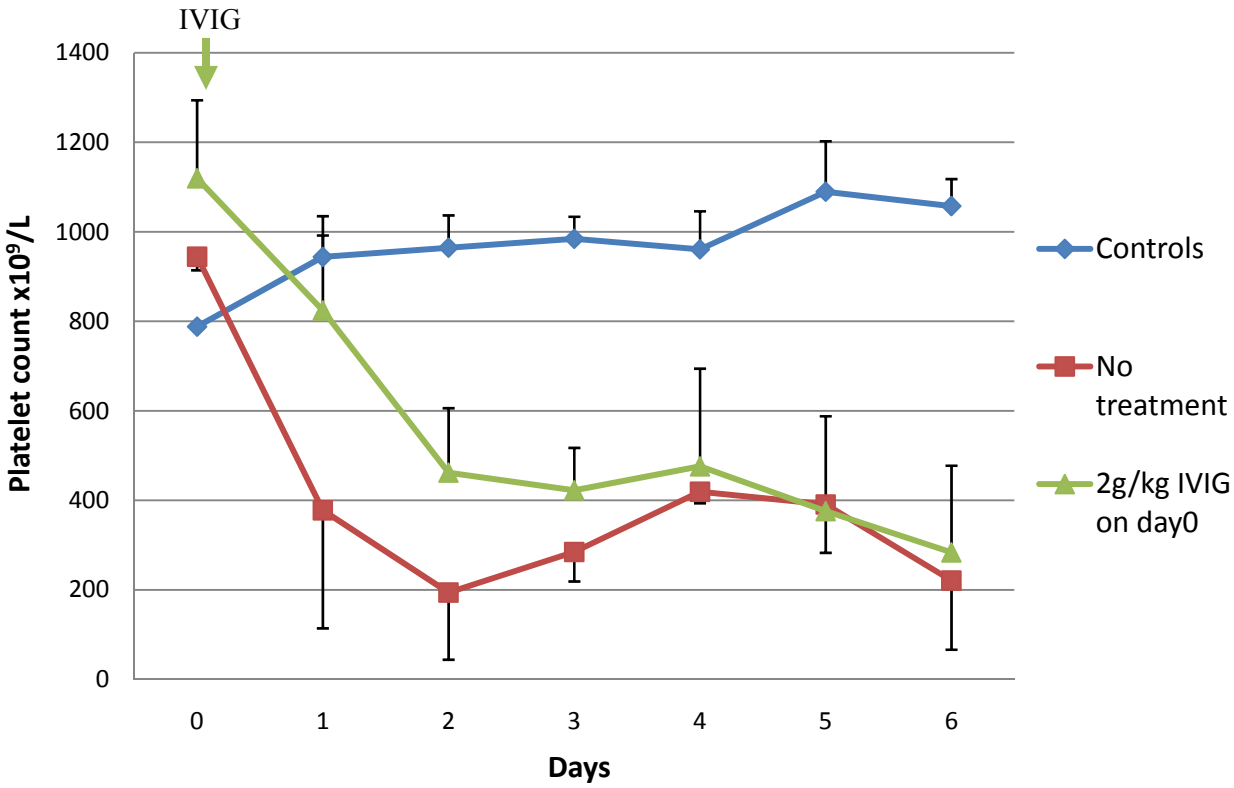
Appendix II.3 Efficacy of 2,4-dinitrophenyl methyl disulfide (C7) using the dose escalation mouse model of PIT in Balb/c mice. All compounds were administered in 800µl volumes IP on day 2 of the experiment, 2 hours post anti-CD41 administration. Data from different experiments was pooled together, resulting in variability in sample sizes between different groups. Platelet counts of individual mice in each group are shown. **A**. 10% DMSO/PBS. **B**. C7 dissolved in 10% DMSO/PBS by heating overnight at 60C (clear solution) at an approximate maximum concentration of 200mg/kg. **C**. C7 mixed in 10% DMSO/PBS at room temperature (suspension) to an approximate maximum concentration of 400mg/kg. This data constitutes the mean platelet counts shown in Figure 3.6.3A. Please refer to Table 2.5.3.2 for detailed information on the preparation of compounds.

Appendix II.4 – Efficacy of second generation sodium salts when administered IP to Balb/c mice



Appendix II.4 Efficacy of second generation sodium salts using the dose escalation mouse model of PIT in Balb/c mice. All compounds were administered in 800µl volumes IP on day 2 of the experiment, 2 hours post anti-CD41 administration. Data from two different experiments was pooled together, and platelet counts of individual mice in each group are shown. **A**. 10% DMSO/PBS. **B**. *p*-nitrophenyl *ω*-hydrocarboxymethyl disulfide (C10) in 10% DMSO/PBS (approximate maximum concentration = 120mg/kg). **C**. *p*-nitrophenyl *ω*-hydrocarboxyethyl disulfide (C11) in 10% DMSO/PBS (approximate maximum concentration = 120mg/kg). This data constitutes the mean platelet counts shown in Figure 3.6.3B. Please refer to Table 2.5.3.2 for detailed information on the preparation of compounds.

Appendix III – IVIG administration on day 0, delays the anti-CD41 induced drop in platelet counts



Appendix III IVIG efficacy using the TBI-combination model in CD1 mice. All test mice received 1Gy TBI on day 0, as well as daily injections of 68µg/kg anti-CD41 antibody. Mice in the treatment group received 2g/kg of IVIG IP on day 0 of the experiment. n=3 mice with bars representing standard deviations.

Appendix IV

University Health Network

Toronto General Hospital Toronto Western Hospital Princess Margaret Hospital

Animal Care Committee

January 19th, 2008

Dr. Donald R Branch

Dear Dr. Branch:

Re: **AUP # 829.15 - The efficacy of intravenous administration of chemical compounds used at maximum tolerated dose to reverse immune mediated platelet destruction in a mouse model of immune thrombocytopenic purpura.**

The University Health Network, Animal Care Committee has approved your Animal Use Protocol Addendum.

Date of Approval: December 18th, 2008

Please note the **AUP #829.15** must be quoted when placing animal orders with the Animal Resources Centre. Animal ordered under this protocol must only be used for this approved project and are subject to the space availability in the vivarium.

This protocol will expire on 2009-09-19

Sincerely,

Dr. Jeffrey A. Medin, PhD
Chairperson, Animal Care Committee
University Health Network, TMDT/TGH/TWH/CBS

Please note: All Animal Care related correspondences should be addressed to: ARC, c/o Lih Ling Chung, 6th Floor, Rm. 6-902, MaRS – Toronto Medical Discovery Tower, 101 College Street, Toronto, Ontario, M5G 1L7.