Anti-RBC antibody mediated erythrocyte phagocytosis and its therapeutic role in immune thrombocytopenia (ITP)

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Laboratory Medicine and Pathobiology
University of Toronto

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2016

Abstract

Immune thrombocytopenia (ITP) is a platelet autoimmune disorder diagnosed by an isolated low platelet count and the absence of other causes of thrombocytopenia. Rh immune globulin (anti-D) is a first line therapy for ITP which targets the RhD antigen on erythrocytes. Monoclonal antibodies are desirable alternatives to anti-D for various reasons. Here, nine erythrocyte-specific antibodies were evaluated for their ability to opsonize RBCs for phagocytosis and prevent platelet destruction in vitro using these opsonized RBCs. Only three (34-3C, TER119 and M1/69) effectively induced phagocytosis of RBCs. Antibodies that were able to opsonize RBCs for phagocytosis also inhibited platelet phagocytosis. TER119, 34-3C and M1/69 are the only antibodies tested that ameliorate ITP in a passive mouse model. These data suggest that the ability of an antibody to prevent platelet phagocytosis in vitro could be indicative of its ability to ameliorate ITP. We speculate that a future screening tool to test the therapeutic ability of monoclonal antibodies in ITP may be possible using these methods.
Acknowledgements

I want to begin by thanking Mark Jen for taking me in as his mentee in the lab, his knowledge and patience was greatly appreciated while learning new techniques in the lab. I would also like to thank Dr. Alaa Amash for teaching me the phagocytosis protocols and also helping me become acquainted with the flow cytometer and confocal microscope in the facility. He was a great help and without him I would not have been able to conduct my experiments.

Thank you to Andrew Crow for carrying out the M1/69 experiments and for always being around to answer my various questions. Thank you to Dr. Ben Yu for collaborating with me on several experiments and generating the deglycosylated TER119 antibody. I want to thank Ms. Joan Legarda for being a calming presence in the lab and making sure I had everything I needed to carry out experiments. Thank you to my other lab members Dr. Lidice Bernardo-Reyes, Danielle Marjoram and Dr. Lin Wang for being a friend and sympathizing with lab frustrations.

I want to thank the members of The St. Michael’s Student Research Association and the CLAMPS council for organizing great events that took my mind away from the lab.

I want to thank my committee members Dr. John Semple and Dr. Greg Fairn for contributing a great deal to my success and timely completion of my masters. I really appreciated the feedback during our meetings, it has helped me grow as a researcher.

Most importantly, I want to thank my supervisor Dr. Alan Lazarus. He has been a great role model and mentor. He always made me feel comfortable to approach him when problems arose in the lab and he was also around to share in the successes. He really takes interest in his student’s success, which created a pleasant environment to in which conduct research. I’m extremely grateful for Alan’s continuous guidance and assistance over the years and feel as though I have gained a mentor for life.
Table of Contents

Contents

Table of Contents ........................................................................................................ iv
Abbreviations ............................................................................................................... ix
List of Tables ............................................................................................................... x
List of Figures ............................................................................................................. xi
Chapter 1 .................................................................................................................... 1
1 IMMUNE THROMBOCYTOPENIA (ITP) ................................................................. 1
   1.1 Pathophysiology of primary ITP ................................................................. 1
      1.1.1 Platelet reactive auto-antibodies ....................................................... 2
      1.1.2 Complement-mediated platelet destruction ...................................... 2
      1.1.3 Abnormalities of T cells ................................................................. 3
      1.1.4 Suppressed platelet production ....................................................... 3
   1.2 Pathophysiology of secondary ITP .............................................................. 4
      1.2.1 Autoimmune disorders ................................................................. 4
      1.2.2 Infectious agents ........................................................................... 4
Chapter 2 ..................................................................................................................... 7
2 THERAPUTIC INTERVENTIONS IN ITP ........................................................... 7
   2.1 First-line treatments for ITP ..................................................................... 7
      2.1.1 Corticosteroids .............................................................................. 7
      2.1.2 Intravenous immunoglobulin (IVIg) ............................................... 8
      2.1.3 Rh immune globulin (anti-D) ....................................................... 9
   2.2 Second-line treatments for ITP ................................................................. 10
      2.2.1 Splenectomy .................................................................................. 10
      2.2.2 Anti-CD20 antibody (Rituximab) ................................................. 10
2.2.3 Thrombopoietin receptor agonists (TPO-RAs) ............................................... 11

Chapter 3 ......................................................................................................................... 12

3 THE STUDY OF ANTI-D AND RELATED THERAPIES ............................................. 12

3.1 Anti-D in hemolytic disease of fetus and newborn (HDFN) ................................. 12

3.2 Anti-D in ITP ............................................................................................................. 13

3.2.1 Disadvantages and limitations of using polyclonal anti-D in treating ITP......... 13

3.2.2 Recombinant monoclonal antibody therapy in the treatment of ITP .............. 14

3.3 Mechanisms of anti-erythrocyte antibodies in the amelioration of (murine) ITP .... 15

3.3.1 Cytokine modulation ......................................................................................... 16

3.3.2 Activating and inhibitory FcγRs .................................................................... 16

3.3.3 Mononuclear phagocytic system (MPS) blockade ....................................... 17

3.3.4 Hypoxic anemia ............................................................................................... 18

Chapter 4 ......................................................................................................................... 20

4 RATIONALE AND HYPOTHESIS ............................................................................. 20

4.1 Brief Background .................................................................................................... 20

4.2 Rationale ................................................................................................................ 20

4.3 Hypotheses ............................................................................................................. 21

Chapter 5 ......................................................................................................................... 22

5 MATERIALS AND METHODS .................................................................................. 22

5.1 Mice ......................................................................................................................... 22

5.2 Reagents ................................................................................................................. 23

5.3 Platelet counting .................................................................................................... 25

5.4 Erythrocyte counting ......................................................................................... 26

5.5 Antibody injections .............................................................................................. 26

5.6 Induction of passive immune thrombocytopenia (ITP) ....................................... 26
5.7 Treatment of ITP with anti-erythrocyte antibodies ............................................. 26

5.8 Erythrocyte phagocytosis assay ............................................................................. 27
  5.8.1 Preparation of RAW264.7 cells ....................................................................... 27
  5.8.2 Erythrocyte enumeration .................................................................................. 27
  5.8.3 Opsonisation of RBCs with anti-RBC antibodies ............................................. 27
  5.8.4 Incubation with RAW264.7 cells ..................................................................... 28
  5.8.5 Fixation of Cells ............................................................................................... 28
  5.8.6 Calculation of the Phagocytic Index ................................................................. 28

5.9 Immunofluorescence detection of opsonized erythrocytes .................................. 28

5.10 Erythrocyte to macrophage binding assay ......................................................... 29

5.11 Active inhibition assay ....................................................................................... 29

5.12 Platelet phagocytosis assay ................................................................................ 29
  5.12.1 Preparation of RAW264.7 cells ..................................................................... 29
  5.12.2 Platelet and Erythrocyte enumeration ............................................................. 30
  5.12.3 Labeling of platelets with CMFDA Cell Tracker Green ................................. 30
  5.12.4 Opsonisation of platelets with anti-CD41 (Mwreg30) antibody and of RBCs with anti-RBC antibodies ................................................................. 30
  5.12.5 Incubation with RAW264.7 cells .................................................................. 31
  5.12.6 Post phagocytosis preparation ...................................................................... 31
  5.12.7 Confocal imaging and calculation of the Phagocytic Index .......................... 31

Chapter 6 .................................................................................................................. 32

6 RESULTS .................................................................................................................. 32

  6.1 Erythrocyte-specific antibodies in RBC clearance and amelioration of murine ITP ...... 32
    6.1.1 Anti-OVA polyclonal antibody ...................................................................... 33
    6.1.2 Anti-hGPA antibody 6A7 ............................................................................. 37
    6.1.3 Anti-CD24 antibody .................................................................................... 40
6.2 Erythrocyte-specific antibodies and mediation of RBC phagocytosis in vitro................. 40

6.2.1 RBCs sensitized with anti-mGPA antibody (TER119) are phagocytosed by
RAW macrophages ........................................................................................................... 41

6.2.2 RBCs sensitized with antibodies to the HOD molecule are not phagocytosed
by RAW macrophages ...................................................................................................... 44

6.2.3 RBCs sensitized with the anti-hGPA antibody (6A7) are not phagocytosed by
RAW macrophages ......................................................................................................... 47

6.2.4 RBCs sensitized with the anti-band-3 antibody (34-3C) are phagocytosed by
RAW macrophages ......................................................................................................... 49

6.2.5 RBCs sensitized with the anti-CD24 antibody (M1/69) are phagocytosed by
RAW macrophages ......................................................................................................... 51

6.3 Examination of the discrepancies of various antibodies to cause phagocytosis........... 53

6.3.1 Effect of the erythrocyte to macrophage ratio on phagocytosis ............................. 53

6.3.2 HOD-specific antibody binding to HOD erythrocytes ............................................ 54

6.3.3 Opsonized erythrocyte-macrophage binding assay .................................................. 55

6.3.4 MIMA29’s ability to inhibit phagocytosis of TER119 opsonized RBCs .............. 57

6.4 Assessment of antibody opsonized erythrocytes to prevent platelet phagocytosis in
vitro ..................................................................................................................................... 58

6.4.1 Platelet phagocytosis ............................................................................................. 59

6.4.2 TER119 opsonized RBCs prevent platelet phagocytosis in vitro ....................... 62

6.4.3 Some anti-RBC antibodies can prevent platelet phagocytosis in vitro .......... 65

Chapter 7 .......................................................................................................................... 68

7 DISCUSSION .................................................................................................................. 68

7.1 The relationship between in vivo RBC clearance and in vitro phagocytosis of
opsonized RBCs ................................................................................................................. 68

7.2 Anti-duffy antibodies’ did not promote opsonized HOD-RBC phagocytosis despite
their ability to induce anemia ............................................................................................ 69

7.3 Anti-erythrocyte antibodies’ therapeutic ability is related to inhibition of platelet
phagocytosis .......................................................................................................................... 72

Chapter 8 .......................................................................................................................... 74
8 Summary .................................................................................................................................. 74
Chapter 10.................................................................................................................................. 75
9 Future Directions...................................................................................................................... 75
REFERENCES ................................................................................................................................. 76
Appendix....................................................................................................................................... 84
10 Supplementary Figures............................................................................................................. 84
Abbreviations

AMIS: Antibody mediated immune suppression
Anti-D: Rhesus D immune globulin
BSGC: Buffered Saline Glucose Citrate
CMFDA: 5-Chloromethylfluorescein Diacetate
CPDA: Citrate-phosphate-dextrose solution with adenine
DIC: Disseminated intravascular coagulation
EDTA: Ethylene diamine tetra acetic acid
FcγR: Fcγ receptor
FDA: Food and Drug Administration
HCV: Hepatitis C virus
HDFN: Hemolytic disease of fetus and newborn
HEL: Hen egg lysozyme
HIV: Human immunodeficiency virus
HOD: Hen egg lysozyme- Ovalbumin- Duffy
H pylori: Helicobacter pylori
IgG: Immunoglobulin G
IL-1Ra: IL-1 receptor antagonist
ITP: Immune thrombocytopenia
IVIg: Intravenous Immunoglobulin G
MFI: Mean fluorescent intensity
MPS: Mononuclear phagocytic blockade
OVA: Ovalbumin
PBS: Phosphate buffer saline
PRP: Platelet rich plasma
RES: Reticuloendothelial system
RhD: Rhesus D
R-PE: R-Phycoerythrin
Treg: T regulatory cell
Th: T helper cell
TPO-RAs: Thrombopoietin receptor mimetics
List of Tables

Table 1. List of anti-erythrocyte antibodies ................................................................. 22

Table 2. Buffered Saline Glucose Citrate (BSGC) Recipe ........................................... 25

Table 3. Anticoagulant Buffer recipe ............................................................................ 25

Table 4. Previously evaluated anti-erythrocyte antibodies .......................................... 33

Table 5. Anti-erythrocyte antibodies used in RBC phagocytosis .................................. 41

Table 6. Anti-erythrocyte antibodies ability to mediate RBC phagocytosis ................. 53

Table 7. Anti-erythrocyte antibodies used to inhibit opsonized platelet phagocytosis ... 59

Table 8. Summary of anti-erythrocyte antibody properties .......................................... 67
## List of Figures

- **Figure 1.** Mononuclear system blockade by antibody-opsonized erythrocytes. .......................... 18
- **Figure 2.** Hen egg lysozyme-Ovalbumin-Duffy (HOD) erythrocyte model. ............................... 23
- **Figure 3.** In vitro flow cytometric analysis of HOD erythrocytes with HOD specific antibodies. 34
- **Figure 4.** Effects of anti-OVA IgG on erythrocyte clearance in HOD mice. ............................... 35
- **Figure 5.** Effects of anti-OVA IgG on the amelioration of passive ITP in HOD mice. ............... 36
- **Figure 6.** 6A7 RBC and platelet clearance in hGPA mice. ......................................................... 38
- **Figure 7.** Effects of antibody (6A7) on the amelioration of passive ITP in hGPA mice. ........ 39
- **Figure 8.** Effects of antibody (M1/69) on the amelioration of passive ITP in C57BL6 mice. ..... 40
- **Figure 9.** Dose dependent phagocytic index of TER119 opsonized RBCs ................................. 42
- **Figure 10.** In vitro phagocytosis of TER119 opsonized erythrocytes. ....................................... 43
- **Figure 11.** RBCs opsonized with deglycosylated TER119 are not phagocytosed ..................... 44
- **Figure 12.** Erythrocytes opsonized with HOD specific antibodies are not phagocytosed in vitro. ........................................................................................................................................ 46
- **Figure 13.** Human glycophorin A specific anti-erythrocyte antibody’s inability to induce RBC phagocytosis in vitro. ........................................................................................................ 48
- **Figure 14.** Anti-band 3 antibody (34-3C) can induce RBC phagocytosis in vitro. ..................... 50
- **Figure 15.** Anti-CD24 antibody can induce RBC phagocytosis in vitro. ................................. 52
- **Figure 16.** Macrophage to erythrocyte ratio does not increase phagocytosis ......................... 54
- **Figure 17.** HOD specific antibody’s abilities to bind HOD RBCs. ............................................. 55
- **Figure 18.** Ability of opsonized erythrocytes to bind to macrophages ................................. 57
Figure 19. Inhibition of phagocytosis of TER119 opsonized RBCs using MIMA29 opsonized RBCs. .......................... 58

Figure 20. CMFDA or MWreg30 alone are not sufficient to observe platelet phagocytosis. ...... 60

Figure 21. Phagocytosis of MWreg30 and Nit G opsonized platelets................................. 61

Figure 22. TER119 opsonized RBCs inhibit platelet phagocytosis in vitro ....................... 63

Figure 23. Phagocytic index of platelets incubated with TER119 opsonized erythrocytes....... 64

Figure 24. Anti-erythrocyte antibody coated RBCs ability to inhibit platelet phagocytosis. ...... 66

Supplemental Figure 1. Anti-OVA IgG dose response study in HOD mice. ......................... 84

Supplemental Figure 2. Anti-HEL antibody dose-response studies in HOD mice................. 85
Chapter 1

1 IMMUNE THROMBOCYTOPENIA (ITP)

Immune thrombocytopenia (ITP) is a platelet autoimmune disorder that is associated with increased platelet destruction and/or decreased platelet production\(^1,2\). Increased platelet destruction is believed to be caused by the production of platelet-reactive autoantibodies that results in reduced platelet counts due to platelet clearance through Fc\(\gamma\) receptor-mediated phagocytosis\(^1\). Opsonized platelets are cleared from the circulation by phagocytic cells (splenic macrophages) residing in the mononuclear phagocytic system (MPS)\(^2\). ITP is a rare condition that occurs in approximately 9.5 people per 100,000\(^3\).

ITP is currently a diagnosis of exclusion, meaning that a diagnosis is only made when all the other causes of thrombocytopenia have been excluded and there is no patient history that would indicate normally low platelet counts\(^2\). ITP that occurs in patients without any known underlying illnesses or medical conditions is classified as primary ITP whereas ITP that occurs as a result of or in association with other diseases is classified as secondary ITP\(^4\). ITP is usually characterized by a platelet count below 100x10\(^9\)/L, although the symptoms of ITP can vary in clinical presentation\(^5,6\). Some patients experience recurrent nose bleeds (epistaxis), petechiae (small red dots due to bleeding under the skin) or other bleeding manifestations. The dominant clinical manifestation of ITP that is alarming to both patients and physicians is excessive bleeding. Some patients, at platelet counts of 20x 10\(^9\) platelets/L, will experience a variety of complications whereas others with higher platelet counts will rarely bleed.\(^7\) In the vast majority of cases it is highly unlikely that ITP will become life threatening and the risk of mortality is low\(^8\). Despite this, significant bleeding and severe hemorrhage can still be fatal and should be taken seriously when considering treatment options.

1.1 Pathophysiology of primary ITP

The etiology of primary ITP is still under investigation despite much research\(^2,9,10\). It is challenging to identify the initial pathogenic mechanism due to factors such as the possible brevity of the potential provoking event and the difficult task of recognizing and diagnosing ITP early on in its course\(^2\). In the majority of cases, the cause of primary ITP is considered to be quite complex and it is believed by some to be triggered by unbalanced immune responses and a lack
of immune tolerance which can generally be traced to abnormalities in cell-mediated and humoral immunity.\(^2,4,7,11\)

### 1.1.1 Platelet reactive auto-antibodies

The most significant explanation that currently exists for increased platelet destruction in ITP patients is the production of platelet-reactive autoantibodies from auto-reactive B cells.\(^7,11,12\)

Opsonized platelets are then cleared from the circulation through Fc\(\gamma\) receptor-mediated phagocytosis by splenic macrophages residing in the mononuclear phagocytic system (MPS).\(^2,13\)

These immunoglobulin G (IgG) class antibodies react mainly against the epitopes of platelet glycoproteins IIb and IIIa (although reaction to other antigens is also possible (i.e. Ib and IX))\(^11\) and are often detected in patients with ITP.\(^2\) As of yet, the initial event that leads to the production of these autoantibodies in primary ITP is unknown.\(^2\)

As previously mentioned, platelets are thought to be cleared via the MPS once they are bound by anti-platelet auto-antibodies. The constant Fc region of the antibodies can potentially interact with any of the activating Fc\(\gamma\) receptors (Fc\(\gamma\)RI, Fc\(\gamma\)RIIA, and Fc\(\gamma\)RIII) on macrophages. The opsonized platelets are then ingested and degraded in the macrophage. Resident macrophages of the spleen and liver are declared to be the major cell types responsible for Fc\(\gamma\)R-mediated phagocytosis of opsonized platelets.\(^14\)

The leading mechanism of platelet clearance by autoantibodies is thought to be achieved by an Fc\(\gamma\)R-dependent pathway however, it has been demonstrated that anti-GPIb antibodies, as opposed to anti-GPIIbIIIa, can induce thrombocytopenia in an Fc-independent manner.\(^7,15\)

### 1.1.2 Complement-mediated platelet destruction

In addition to Fc\(\gamma\)R-mediated phagocytosis, complement-mediated platelet destruction by auto anti-platelet antibodies may also be involved. In vitro studies using the serum of ITP patients have shown that it is possible for complement to bind to platelets and activate the complement cascade.\(^16,17\)

Sensitization of platelets, using serum from ITP patients, demonstrated detectable complement components (C3, C4, and C9) and the amount of complement on the surface of platelets was positively correlated with the amount of antiplatelet antibody in the sera.\(^16\)

The role complement plays in the reduction of platelets in ITP is still not clear. Based on the current literature, it is possible that complement-mediated platelet destruction is an alternative mechanism in which platelets can be cleared.\(^2,17,18\)
Therefore, complement is probably associated with decreased platelet survival due to enhanced clearance of opsonized platelets by the MPS in the liver as opposed to the spleen\textsuperscript{11}. In addition, it was found that C5b-9 deposition on target cells can cause cytolysis, and may therefore cause direct platelet damage, and damage to megakaryocytes, the progenitor cell of platelets, thus affecting platelet production\textsuperscript{17}.

1.1.3 Abnormalities of T cells

It has been documented that there exist differences in the phenotypes of T cells in ITP patients and can affect the type of responses that the T helper cells generate\textsuperscript{2,6}. CD4\textsuperscript{+} T helper cells are generally divided into two subsets Th1 (e.g. interferon producers) and Th2 cells (e.g. IL4 producers) which differ in the types of cytokines they produce. The Th1/Th2 balance plays an important role in the regulation of the immune system, and is known to be compromised in many autoimmune diseases including ITP\textsuperscript{19}. The ratio of Th1 to Th2 responses appears to be correlated with the severity of disease, in that as the ratio increases the platelet count decreases\textsuperscript{2}. Once inflammation begins to subside, the Th1 and Th2 balance is restored. Th cells aren’t the only ones to be dysregulated in ITP patients, many other sub-types of T cells are also found to be unbalanced.

T-regulatory cells (Tregs), whose function is to down-regulate T-cell responses, have lower numbers in ITP patients and become even lower as the disease progresses\textsuperscript{2,20}. Because this cell type plays an important role in regulating the immune system by suppressing excess reactions from taking place during an inflammatory event, the loss of Treg cell populations or activity may have potential implications in the development of ITP and other autoimmune diseases. In addition, Th17 (CD4\textsuperscript{+}) cells, T cells which secrete IL-17, are increased in ITP\textsuperscript{21}. These cells are pro-inflammatory and of interest in ITP in part due to a large body of evidence implicating IL-17 in autoimmunity\textsuperscript{1,20,22,23}.

1.1.4 Suppressed platelet production

In vivo kinetic studies have shown that there is a reduction in platelet production in a significant number of patients with ITP which could explain why some patients are refractory to splenectomy or therapy directed at decreasing platelet clearance\textsuperscript{7,24}. Anti-platelet antibodies present in the plasma of ITP patients have been shown to be reactive to megakaryocyte glycoproteins which could impair megakaryocyte development or impede platelet release.
depending on the targeted antigen. In vitro studies have shown that this inhibits megakaryocyte development. As mentioned above, complement fixation may also have an effect on megakaryocyte health and platelet production. Other mechanisms may be involved in lower platelet production such as cytotoxic T cells, genetic or acquired factors that could limit megakaryocyte and platelet production however these remain unexplored.

1.2 Pathophysiology of secondary ITP

Secondary ITP occurs as a side effect of another associated disease or medical condition and can account for up to 20% of patients with ITP. Typically, these patients experience complete remission once the inciting agent is removed. Primary and secondary ITP present as a significant reduction in platelet counts, however the causes of secondary ITP are diverse, and each form of secondary ITP may have its own unique immunologic profile.

Seeing as there are many causes and intricacies of secondary ITP which are beyond the scope of this thesis only a brief review will be presented.

1.2.1 Autoimmune disorders

There exist a vast number of autoimmune disorders which lead to extensive dysregulation of the immune system. As expected, some of these are able to cause thrombocytopenia including: Evans syndrome, systemic lupus erythematosus, some thyroid diseases, antiphospholipid syndrome and lymphoproliferative disorder. Because these diseases can exhibit multiple known and unknown immunodeficiencies, the mechanism used by these disorders to cause thrombocytopenia could be more complicated than what is observed in primary ITP. This makes the pathogenesis of secondary ITP quite complex to study.

1.2.2 Infectious agents

Human immunodeficiency virus (HIV), helicobacter pylori (H. pylori), and hepatitis C virus (HCV) are known infectious agents associated with secondary ITP.

1.2.2.1 Human immunodeficiency virus (HIV)

HIV infects thymic lymphocytes, monocytes, macrophages and megakaryocytes by interacting with a glycoprotein found on the surface of immune cells known as CD4 (cluster of differentiation 4). HIV is believed to cause thrombocytopenia by infiltrating the bone marrow
and infecting megakaryocytes by binding to the CD4 receptors on their surface. As the virus replicates within the cells it causes abnormal growth and differentiation of infected cells which can ultimately lead to cell death. HIV antibodies have been shown to be able to cross-react with immune complexes and platelet-membrane glycoproteins which can activate the host immune system to destroy platelets, a phenomenon known as molecular mimicry. Early HIV infection associated thrombocytopenia is more frequently characterised by increased destruction of platelets, whereas in patients who have progressed to develop immunologic AIDS, thrombocytopenia is caused mostly due to decreased platelet production.

1.2.2.2 Hepatitis C virus (HCV)

There exists a strong relationship between the development of thrombocytopenia and infection with HCV. The exact cause for this relationship is not yet fully understood, however a variety of pathogenic mechanisms have been hypothesized. For one, HCV is known to be able to bind human CD81 receptor directly on the platelet membrane which causes autoantibody production against this complex and leads to increased platelet destruction. HCV, similar to HIV, may also cause thrombocytopenia by infecting megakaryocytes directly thus decreasing platelet production. As the disease progresses, HCV can lead to liver damage, impaired thrombopoietin production, portal hypertension and splenomegaly which can also lead to decreases in platelet numbers.

1.2.2.3 Helicobacter pylori (H. Pylori)

H. pylori is a gastrointestinal bacterium that can cause several pathologies in humans including chronic inflammation, ulcers, cancer and in some cases ITP. The immune component of H. Pylori associated ITP may be mediated through molecular mimicry involving antibodies to the H. Pylori Cag A antigens. Moreover, some strains of H pylori can induce platelet aggregation and platelet expression of p-selectin and phosphatidylserine. Genetic factors such as Lewis or HLA type are also linked to H pylori– associated ITP.

During H pylori infection, T cells are generally hyporesponsive which results in a T helper 1 (Th1) skewed response, characterized by high levels of interferon-γ and tumor necrosis factor-α production. H pylori neutrophil-activating protein (HP-NAP) and the cell wall lipopolysaccharide (LPS) are thought to play a role in this response. LPS has been shown to promote Th1 type immune response in immunized BALB/c mice that may aid in the protection
or clearance of *H pylori* infection. It was also demonstrated that in the presence of antiplatelet antibodies, the LPS of Gram negative bacteria can significantly enhance Fc-dependent platelet phagocytosis\(^\text{30}\).

Therefore, there appear to be a variety of factors involved in *H pylori*-associated ITP. These include the capacity for molecular mimicry, susceptibility to the infection and variation in the ability of the host to present of bacterial peptides to the immune system\(^\text{7}\). The successful management of *H. Pylori*-associated ITP is ultimately dependent on the efficiency of anti-bacterial therapies that are used today to eradicate the *H. Pylori* in that specific region\(^\text{2}\). 
Chapter 2

2 THERAPEUTIC INTERVENTIONS IN ITP

In most cases of ITP, symptoms are non-life threatening however, severe thrombocytopenia can be dangerous due to risks of heavy internal bleeding caused by trauma or surgery. Since the symptoms of ITP can vary in clinical presentation, the primary factors considered in deciding the treatment course of a particular patient are platelet counts and bleeding symptoms\(11\). Treatment is often not required in patients with platelet counts above \(50 \times 10^9\) platelets/L who do not present with bleeding or have any comorbid conditions. In the case that treatment is required, a variety of therapeutic options are available, although treatment is very personalized. First-line therapies of ITP include corticosteroids, intravenous immunoglobulin (IVIg), and intravenous RhD immune globulin (IV anti-D, or just anti-D) and second-line therapies include splenectomy, the anti-CD20 therapeutic Rituximab, and thrombopoietin receptor mimetics (TPO-RAs)\(^4,11,31,32\).

2.1 First-line treatments for ITP

2.1.1 Corticosteroids

When an initial diagnosis of immune thrombocytopenia is made, often corticosteroids are the initial treatment recommended in adults and children based on published guidelines\(^5,10,31,33\). Corticosteroids are group of natural and synthetic analogues of the hormones secreted by the adrenal gland. Prednisone and dexamethasone are the most common corticosteroids used in ITP and are from the subclass known as glucocorticoids, which help regulate the immune system by suppressing the production of a multitude of cytokines\(^34,35\). When corticosteroids interact with the glucocorticoid receptor on cells, they can regulate the transcription of various genes and thus inhibit the downstream effects of many cell types including B cells and T cells. Corticosteroids can induce T cell apoptosis, suppress T cell proliferation, inhibit T cell signaling, and reduce phagocytosis\(^35\). However, due to the myriad of effects of corticosteroids, prolonged treatment regimens are not without risk\(^31,35\) The long term use of corticosteroids is associated with a number of adverse health effects including growth impairment in children, increased risk of infection, obesity, diabetes and osteoporosis\(^36\). Despite their widespread use in a multitude of different autoimmune diseases, it’s still unclear how these steroids suppress the immune system to ameliorate ITP and why some patients are refractory to these treatments. It has been suggested
that the efficacy of steroid therapy may be dependent on the dominant type of anti-platelet autoantibodies that are mediating the disease\textsuperscript{37}. Steroids have a very broad role in regulating homeostasis and as a result it is very difficult to study exactly how they are able to improve ITP.

2.1.2 Intravenous immunoglobulin (IVIg)

Intravenous Immunoglobulin (IVIG), a donor pooled preparation consisting of mostly IgG antibodies, was first used successfully for the treatment of ITP in 1981 by the Imbach group\textsuperscript{10}. IVIg is effective in treating many immune-mediated and inflammatory diseases. In ITP patients, an increase in platelet counts can be observed as early as 24-48 hours after treatment\textsuperscript{4}. IVIg could be considered safer than corticosteroids and has a better response rate however, IVIg is very costly due to the production costs and high therapeutic dosage (1-2 g IgG/kg body weight)\textsuperscript{38}. Although rare, serious infusion reactions may occur during infusion with IVIg\textsuperscript{4,39}. In addition, since IVIg is a blood-derived product, IVIg treatment has the theoretical potential for transmitting infectious diseases (HIV, HCV, etc.) as well as non-conventional infectious agents\textsuperscript{11,34}. The concern of infectious agents do not lie in existing pathogens but with emerging pathogens. The introduction of more tests to detect potential emerging pathogens increases the cost and complexity of these products.

The exact mechanism(s) of how IVIg is able to increase platelet counts is not fully understood although various hypotheses exist. Some of the proposed mechanisms include: increased expression of the inhibitory FcγRIIB on macrophages, the presence of anti-idiotype antibodies in the preparation, immunomodulatory effects, apoptosis of lymphocytes, cytokine activation, complement, and activation of dendritic cells\textsuperscript{32}. The first demonstration that IVIg anti-inflammatory activity was through effects on dendritic cells was done by the Lazarus lab\textsuperscript{40}. There is a growing body of evidence that supports the action of IVIg on dendritic cells\textsuperscript{34,40}.

IVIg has been shown to inhibit in vitro dendritic cell activation and eliminate auto-reactive T cell activation through down-regulation of costimulatory molecules\textsuperscript{41}. Moreover, the expression of murine SIGN-R1, an orthologue of the human dendritic cell-specific intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), was found to be required for IVIg to demonstrate its protective effect in murine ITP. DC-SIGN may play a significant role in self-antigen recognition and tolerance\textsuperscript{42}. Although, some work has argued against a role for DC-SIGN\textsuperscript{43}. There is also recent evidence being brought towards the role of tregitopes in the action
of IVIG. Tregitopes are amino acid sequences that are highly conserved in Fc and Fab regions of human IgG antibodies which demonstrate high affinity binding to multiple HLA class II alleles and can activate natural regulatory T cells.\textsuperscript{44} It is thought by some that IVIg acts in part due to T cells.\textsuperscript{4,39} Tregitopes provide one explanation for the expansion and activation of Treg cells following IVIg treatment. Tregitopes’ effects may possibly be similar to IVIg in vitro in suppressing immune response although this has yet to be shown. The discovery of Tregitopes in IgG and other autologous proteins may contribute to improved understanding of the mechanism of action of IVIg. More research is required to fully understand the mechanism(s) of IVIg action in ITP. Although dendritic cells have certainly taken center stage in explaining how IVIg may work.

### 2.1.3 Rh immune globulin (anti-D)

The first successful use of anti-D in the treatment of ITP was in 1983 by Salama and colleagues.\textsuperscript{45} Since this key observation, intravenous anti-D has become one of the first-line treatments of ITP in both adults and children. Anti-D is a pooled plasma product from repeatedly immunized RhD\textsuperscript{-} donors that targets the RhD antigen on erythrocytes and is therefore a polyclonal antibody. It was initially used for the prevention of hemolytic disease of the newborn (HDN), however it has been successfully used in the treatment of ITP since 1983.\textsuperscript{45} Since the first use of anti-D in 1983 several experiments have been conducted to determine the effectiveness, the toxicity as well as the mechanism of action.\textsuperscript{46,47,48} The mechanism of anti-D is still highly speculative, the principal theory is that anti-D functions via mononuclear phagocytic system (MPS) blockade. Based on this theory, sensitized erythrocytes bind to Fc gamma receptors (Fc\textgammareceptors) on macrophages and initiate phagocytosis thus competitively inhibiting platelet destruction.

Anti-D is the preferred treatment in ITP by some because it is less expensive (14\% of IVIg cost)\textsuperscript{38} and is associated with a decreased risk of secondary symptoms such as viral infection, aseptic meningitis, headache, and vomiting as compared to IVIg.\textsuperscript{38} Anti-D is also given at a lower therapeutic dosage than IVIg (50-75\textmu g anti-D IgG/kg vs. 1-2g/kg IVIg)\textsuperscript{32}. Anti-D is more expensive than steroids, however it is often preferred in children because of the toxicity of steroids and other complications that can arise.\textsuperscript{49} Despite these benefits, polyclonal anti-D is in limited supply and its widespread use in ITP patients would not be possible as a result of the priority given to the prevention of alloimmunization in pregnant D- women.\textsuperscript{50} In addition, its
administration can be accompanied by adverse effects such as acute hemolysis and in extreme cases renal failure. Anti-D is also a donor derived product that carries a theoretical risks of infection. Because of this, there is currently a black box warning on the use of anti-D to treat ITP that was put forward by the US Food and Drug Administration (FDA)\textsuperscript{4}. Despite this black box warning, many hematologists who treat ITP feel that anti-D can still be safely used in ITP\textsuperscript{51}. Anti-D’s mechanism of action and uses will be discussed further in this thesis.

2.2 Second-line treatments for ITP

If first line treatments are not effective in raising platelet counts, then second-line treatments will be considered.

2.2.1 Splenectomy

The spleen is believed to be the primary site of circulating platelet destruction by antibody recognition by Fc receptors on macrophages within the mononuclear phagocytic system. Therefore the removal of this organ removes the main site of platelet recognition and clearance. More than two-thirds of patients who undergo splenectomy will experience a sustained remission of ITP symptoms for a minimum of 5-10 years following the procedure\textsuperscript{52}. However, due to the invasive nature of the surgery there can be complications following surgery such as the loss of immune protection against encapsulated organisms, thrombosis and sepsis as a result of surgical complications\textsuperscript{11}. To counteract this, immunizations against encapsulated bacteria are recommended two weeks before surgery and the development of laparoscopic surgical techniques have reduced surgical complications significantly. Despite splenectomy having a high success rate, there are increasing number of therapeutic options being developed which has caused physicians and patients to be reluctant to recommend splenectomy for patients with chronic ITP\textsuperscript{4,11}. Although the remission rate is high, there is always a chance of relapse among some patients.

2.2.2 Anti-CD20 antibody (Rituximab)

Rituximab, a chimeric monoclonal anti-CD20 antibody, causes rapid depletion of CD20 positive B cells by FcγR-mediated destruction\textsuperscript{52–54}. It is utilized to treat numerous immune system diseases that are characterized by an abundance of B cells or dysfunctional B cells, for example, lymphoma and rheumatoid arthritis\textsuperscript{52,54}. Reviews of clinical studies have shown that Rituximab
can induce a partial platelet count response (platelets >50x10^9/L) in refractory ITP patients that can last up to 2 years^{54,55}.

There is much to learn about the mechanism of ITP when studying how Rituximab can treat ITP. In spite of the fact that the pathogenesis of ITP is contributed to by platelet auto-antibodies that involve B cells, a noteworthy number of patients won't react to Rituximab and not all whom reacted will support a long term remission^{52}. In light of this, there have been reports recommending that Rituximab's impacts may be essentially reliant on changes to the T cell compartments as a result of the removal of pathogenic B cells^{55,56}. Restoring the Th1/Th2 ratio may be required for Rituximab to demonstrate a positive impact in ITP. Furthermore, for patients that failed to react to Rituximab, B cell screening revealed that antiplatelet-specific plasma cells kept on persevering in the spleen^{57}. This discovery suggests that there could be hereditary varieties among the expression of CD20 on B cells or some patients could do not have the immunogenic sensitivity to respond to the treatment.

2.2.3 Thrombopoietin receptor agonists (TPO-RAs)

TPO-RAs are recent therapeutic agents that have shown much promise in the treatment of ITP. They interact with the TPO receptors of megakaryocytes and stimulate the production of platelets^{2,4}. Two TPO-RAs, romiplostim and eltrombopag, have been approved by the FDA for the purpose of treating patients with chronic ITP. TPO-RAs are not essentially thought to be immunomodulatory operators, but rather as thrombopoietin mimetics to encourage platelet release from megakaryocytes^{2,4}. Despite the fact that reports propose TPO-RAs may have the capacity to increase Treg cell activity, consequently showing an immunomodulatory reaction, further research will be required to affirm this hypothesis^{58}. Both romiplostim and eltrombopag have been effective in treating ITP (61% and 59% to 81% reaction rate individually) while long term assessments of persistent treatment uncovered that the platelet response can be maintained as long as the medication re-administered^{59}. There are a couple of concerns in regards to the utilization of TPO-RAs, for example, reticulin formation in the bone marrow and an increased risk of thrombosis^{60}. Also, a Black box warning has been set on the utilization of Eltrombopag by the FDA due to its potential to cause hepatotoxicity^{4}. 
Chapter 3

3 THE STUDY OF ANTI-D AND RELATED THERAPIES

Due to the lack of long term effective therapies for ITP patients there is a growing need to develop better treatments. Corticosteroids are the favored first-line therapy for management of ITP, however, due to the various side effects associated with them; long term treatment is not recommended and can be detrimental in children or patients with immunodeficiency\(^{35,49}\). Steroid therapy also has a more prolonged response and may take longer to increase platelet counts, as a result it is not often used in patients who urgently need to reestablish platelet levels \(^{4,61}\). IVIg and anti-D are also first-line therapies for ITP that can quickly increase platelet counts, however these therapies are expensive and are in limited quantity; both therapies are often reserved only for patients with serious thrombocytopenia\(^{33}\). These therapies are also donor based which means there is always a risk for donor transmitted infections and contaminants. Therefore, there is a need for a more effective and safer treatment. Monoclonal antibodies are increasingly of interest to replace polyclonal anti-D\(^{62,63}\). An advantage of using a recombinant monoclonal antibody therapy is that monoclonal antibodies can be mass produced. This feature can potentially and drastically reduce the cost per treatment of ITP, increase the overall treatment availability, and eliminate risk of plasma contaminants or donor transmitted infections.

3.1 Anti-D in hemolytic disease of fetus and newborn (HDFN)

The primary use of anti-D is the prevention of hemolytic disease of fetus and newborn (HDFN). HDFN occurs when a mother and the baby have incompatible blood types. HDFN can occur when an RhD\(^{-}\) expecting mother is carrying a baby that is RhD\(^{+}\) through transplacental hemorrhage or following feto-maternal hemorrhage at birth\(^{64,65}\). This creates an antibody response from the mother towards the D antigen on fetal RBCs, this response is associated with high infant mortality and miscarriage during pregnancy\(^{64,65}\). Usually, the first child is unaffected however, subsequent pregnancies are at risk because these anti-D antibodies can cross the placenta and bind the fetal D-positive erythrocytes and induce hemolysis\(^{65,66}\). Extensive anemia in the newborn may also lead to edema and jaundice due to elevated levels of bilirubin caused by the excessive breakdown of heme\(^{65,66}\). Although rare, jaundice can lead to complications such as
cerebral palsy, deafness and kernicterus (brain damage from high bilirubin levels) if it is not treated immediately.

Anti-D is administered to RhD- mothers during and after pregnancy to prevent HDFN or D antigen alloimmunization\(^65,66\). The mechanism of anti-D in the prevention of HDFN has been researched extensively however, it is still unclear. Several hypotheses have been suggested and most suspect that anti-D has an ability to suppress the mother’s immune system through antibody mediated immune suppression (AMIS)\(^64\). There exists several theories to explain the AMIS effect with erythrocytes. One such theory suggests that the antibodies are able to rapidly clear the target cells (RBCS), by Fc\(\gamma\)R mediated phagocytosis or other clearance mechanisms, before they can be recognized by the immune system. Another theory is that antibodies mask the epitopes thus preventing an immune response. However these theories have been challenged by work in our lab\(^67,68\). Other theories also exists such as changes in the conformation of the antigen to prevent the immune response, the production of inhibitory cytokines following detection of antibodies and/or different glycosylation of the antibodies\(^67\).

### 3.2 Anti-D in ITP

As previously mentioned anti-D was first used to treat ITP in 1983, since then several experiments have been conducted to determine the effectiveness, the toxicity as well as the mechanism of action\(^46,47,48\). Anti-D is the preferred treatment in ITP by some because it less expensive (14% of IVIg cost \(^38\)) and is associated with a decreased risk of secondary symptoms such as viral infections, aseptic meningitis, headache, and vomiting as compared to IVIg\(^38\). Anti-D is more expensive than steroids, however, it is often preferred in children because of the toxicity of steroids other complication that can arise\(^49\). Despite all of the benefits of using anti-D in ITP there still exists several limitations and an alternative product is highly desirable.

#### 3.2.1 Disadvantages and limitations of using polyclonal anti-D in treating ITP

Although polyclonal anti-D is a very effective treatment for ITP, it has several limitations and negative side effects which impact its usage. To begin with, anti-D is only effective in patients who are RhD\(^+\) and as a result has less versatility than IVIg\(^69\). Second, the primary use for anti-D is for the prevention of HDFN and seeing as there are no alternatives to this therapy at the moment many physicians want to conserve anti-D for this use\(^64–66\). Because anti-D is a donor-
based preparation, there is a finite supply and shortage is always a concern. Third, seeing as anti-D is a donor-derived plasma product, it carries a theoretical risk of transferring infectious diseases\textsuperscript{70}. Although screening of blood products is rigorous, there is a chance that emerging pathogens, which are not on the list of pathogens screened, could theoretically make it through the preparation process and into the product. And fourth, studies have found the use of anti-D is associated with several side effects such as fever, chills and a rare possibility that treatment could lead to the development of disseminated intravascular coagulation (DIC) and acute renal failure if hemolysis becomes severe\textsuperscript{4,33}. As mentioned earlier, the potential for development of DIC has prompted the FDA to add a black box warning to anti-D preparations cautioning against their use, which has since led to a decrease in the usage of anti-D\textsuperscript{51}. Finally, anti-D administration can cause extravascular hemolysis and induce anemia in treated patients\textsuperscript{33,47}.

3.2.2 Recombinant monoclonal antibody therapy in the treatment of ITP

Monoclonal antibodies to RBCs are newly emerging as potential therapies in ITP and are very useful tools to elucidate the mechanisms involved with anti-D administration\textsuperscript{71–74}. They can also be mass produced and administered without risk of plasma contaminants or donor transmitted infections. The use of monoclonal antibodies would allow further study into the mechanism of anti-D in ITP to determine whether or not the adverse events are important in anti-D’s action or whether they could be removed from a future product.

A study performed in 1996 by Godeau et al. demonstrated that a single monoclonal anti-D did not ameliorate ITP\textsuperscript{50}. However, this was only one antibody. New studies have demonstrated that the isotype and epitope specificity of anti-D is important in its effectiveness as a therapeutic agent. For example, a study done by Kjaersgaard et al. demonstrated that IgG\textsubscript{3} antibodies were more effective in preventing platelet phagocytosis than IgG\textsubscript{1} antibodies\textsuperscript{50,73}. This could serve as an explanation to why that particular monoclonal antibody was not effective in ITP patients.

Since then, a new monoclonal antibody mixture known as, Rozrolimupab, has entered clinical trials to determine its safety and clinical effect in ITP patients. Rozrolimupab is a recombinant human monoclonal antibody mixture consisting of 25 different IgG\textsubscript{1} antibodies, targeted against the RhD erythrocyte antigen\textsuperscript{75}. During phase I and II trials, it was determined that this preparation was successful in increasing platelet counts in 62% of Rh\textsuperscript{D+}, non-splenectomized, ITP positive individuals, however, it has many adverse effects. Headache, pyrexia and hematoma
were some of the side effects seen in addition the product was able to cause low platelet counts and even thrombocytopenia\(^6_{2,75}\). Rozrolimupab also caused a drop in hemoglobin level that was greater than that seen with polyclonal anti-D administration and there was also a rise in D-dimers\(^6_{2,75}\). Although Rozrolimupab doesn’t appear to have the best safety profile it does demonstrate that a mixed monoclonal product may be effective in treating ITP. However, due to a small sample size (n=61), the safety profile of Rozrolimupab cannot be fully assessed. Therefore, studies conducted using individual monoclonal antibodies could help determine which antibodies are needed to increase platelet counts, which ones cause erythrocyte phagocytosis/clearance and which ones may cause other side effects.

Work in our lab has demonstrated that TER119, a monoclonal rat IgG\(_{2b}\), that targets mouse glycophorin A on the surface of RBCs, was able to ameliorate ITP in a passive ITP mouse model. However, it caused anemia as early as 24 hrs post injection which peaked in intensity at 4 days post injection\(^7\). It was also observed that there was a positive correlation between the increase in platelet count and the clearance of erythrocytes by therapeutic anti-erythrocyte antibodies in thrombocytopenic mice\(^7\). There are many theories to explain how anti-erythrocyte antibodies ameliorate ITP, these will be explained in the next few paragraphs.

3.3 Mechanisms of anti-erythrocyte antibodies in the amelioration of (murine) ITP

Initially, it was thought that erythrocyte-specific antibodies and IVIg functioned via the same mechanism of action, inhibiting MPS function to prevent immune-mediated platelet destruction\(^4\)\(_{5,76}\). However, this theory has since been argued against based on the work done in the murine models of passive ITP. It was discovered that IVIg is more likely to ameliorate ITP by modifying dendritic cell activity\(^4\)\(_{0,43}\) whereas erythrocyte-specific antibodies are primarily believed to increase platelet counts by inhibiting the MPS function\(^3\)\(_{2,76}\). Other theories suggesting there exists a difference in the primary mechanism of these two therapies include: a different relationship with the inhibitory Fc\(\gamma\)RIIB\(^7\)\(_2\), cytokine profile\(^2\)\(_{0,77,78}\), and the effects on activating Fc\(\gamma\)RIIIA on phagocytic cells\(^7\)\(_2\). The following paragraphs will discuss several potential mechanisms of action of erythrocyte specific antibodies in the amelioration of ITP.
3.3.1 Cytokine modulation

One of the potential mechanisms that have been proposed for how erythrocyte-specific antibodies can increase platelet counts is that they cause changes in cytokine levels which modify the immune system. It has been recorded that in patients receiving anti-D, a variety of cytokines are up-regulated including IL-6, IL-10, TGF-β, TNF-α, MCP-1, and GM-CSF\textsuperscript{78,79}. In addition, IL-1 receptor antagonist (IL-1Ra), an anti-inflammatory cytokine, was significantly increased after anti-D treatment. This finding has been reproduced in vitro using anti-D sensitized erythrocytes in addition it was shown that increases in IL-1Ra was correlated with a decrease in phagocytosis\textsuperscript{78}. As seen with anti-D, a therapeutic monoclonal erythrocyte-specific antibody (TER-119) upon injection into mice was able to increase IL-1Ra as well, however, this antibody was able to ameliorate ITP in mice lacking a functional IL-1Ra system\textsuperscript{80}. Further tests demonstrated that infusion of elevated amounts of recombinant IL-1Ra did not have a beneficial outcome in murine ITP which suggests that IL-1Ra is not required for TER-119 to exhibit a restorative impact\textsuperscript{80}. Although, anti-D can modulate the expression of numerous cytokines in patients with ITP, more research will be required to completely assess the part of cytokines in the remedial action of erythrocyte-specific antibodies.

3.3.2 Activating and inhibitory FcγRs

Fcγ receptors are responsible for inducing phagocytosis of IgG opsonized particles/microbes. In humans there exists several activating receptors (FcγRI, FcγRIIA, FcγRIIC, and FcγRIIIA) and one inhibitory receptor (FcγRIIB). In mice there are three activating receptors (FcγRI, FcγRIII and FcγRIV) and the same inhibitory receptor (FcγRIIB)\textsuperscript{81}. There also exists several differences between human and mouse FcγRs which makes it difficult to study human autoimmune diseases using mice. For one, all human activating FcγRs bind the major human IgG subclass IgG1, whereas only mouse activating FcγRIII binds mouse IgG1. Second, human inhibitory FcγRIIB has a lower affinity for IgG1-3 than other FcγRs, which is not seen in mice for IgG1 and IgG2b. Finally, no human FcγR binds human IgE, whereas FcγRIIB, FcγRIII and FcγRIV in mice bind mouse IgE\textsuperscript{82}. In addition, the cells which express certain FcRs varies between humans and mice. However, due to the ability to modify the expression of Fc receptors in mice, they are a good model to use when studying the mechanism of various therapies.
Two therapeutic monoclonal anti-RBC antibodies, TER-119 and M1/69, have been shown to down regulate the expression of activating FcγRIIIA on splenic macrophages in a murine model of passive ITP\textsuperscript{72}. However, both IVIg and a non-therapeutic antibody (30F1) could not modulate the expression of activating FcγRIIIA\textsuperscript{72}. Therapeutic antibodies to erythrocytes were able to preserve their ameliorative effects in the absence of FcγRIIB inhibitory receptors\textsuperscript{71}. In contrast, IVIg was not able to produce an ameliorative effect in the absence of the inhibitory receptor\textsuperscript{71}. The down modulation of activating FcγRIIIA could cause a variety of downstream effects that hinder immune-mediated platelet destruction which could make it much more complicated than what is seen with the passive ITP model. In addition, TER119, when deglycosylated, did not induce RBC phagocytosis in vitro nor was it able to ameliorate ITP therefore it appears as though Fc regions are required for TER119 to be effective in ITP\textsuperscript{83}.

Seeing as anti-D is a polyclonal product, its effects will also be quite difficult to dissect as it could potentially interact with multiple classes of FcγRs to initiate phagocytosis. The possible down regulation of all classes of activating human FcγRs will have to be examined in mice to definitively answer this question in human patients with ITP.

3.3.3 Mononuclear phagocytic system (MPS) blockade

The most discussed theory on the mechanism of anti-erythrocyte antibodies in ITP is mononuclear phagocytic system (MPS) blockade. This theory suggests that there is competitive inhibition of platelet phagocytosis due to antibody-sensitized erythrocyte’s competition for Fc receptors on splenic macrophages thus resulting in decreased destruction of sensitized platelets within the spleen\textsuperscript{32,43,47,76} (Figure 1). As previously mentioned, this theory was first proposed by Salama and colleagues in 1983 when they successfully used anti-D to treat ITP for the first time. Support for this hypothesis originates from studies exhibiting that anti-D is unable to restore platelet counts in ITP patients who are RhD antigen negative and in patients who continue to experience low platelet counts following splenectomy\textsuperscript{32,84}. In addition, one study showed that patients that were RhD antigen negative, but c antigen positive responded to anti-D treatment\textsuperscript{85}. Even though MPS blockade is the originally proposed mechanism as to how anti-erythrocyte antibodies function, there is very little direct data to actually support this hypothesis. One study did demonstrate that both polyclonal anti-D and select monoclonal anti-D antibodies can prevent phagocytosis of opsonized platelets in vitro and found that both epitope specificity and isotype of
the monoclonal were imperative in determining if prevention of phagocytosis would occur. However, the impact of these studies on clinical outcomes remains unknown.

**Figure 1. Mononuclear system blockade by antibody-opsonized erythrocytes.**

**Left panel.** The suspected mechanism of platelet loss in ITP is that sensitized platelets are cleared by macrophages as they are recognized by Fc receptors. **Right panel.** A depiction of what is suspected to be the major mechanism of action of anti-D. Opsonized erythrocytes are competing for Fc receptors on the surface of macrophages and since erythrocytes greatly outnumber circulating platelets (20:1), macrophages become unable to engulf the sensitized platelets due to competitive inhibition by antibody-opsonized erythrocytes. This is what is recognized as mononuclear phagocytic system (MPS) blockade which leads to increased platelets but also decreased RBCS counts.

### 3.3.4 Hypoxic anemia

Despite the fact that antibody-mediated erythrocyte clearance may assume a noteworthy part in the enhancement of ITP by anti-erythrocyte antibodies due to competitive inhibition, another variable must be considered. Anemia or a decrease in hemoglobin, is observed in ITP patients or mice undergoing treatment with anti-RBC antibodies seeing as erythrocytes are being cleared by macrophages. While trying to maintain blood homeostasis, the body can react to anemia by activating various downstream pathways, for example, an increased production of the hormone
erythropoietin by the kidneys. Clinical trials in patients receiving recombinant human erythropoietin (rHuEpo) therapies have observed significant increases in platelet counts. This has also been observed in other species such as dogs, mice and rats given high doses of rHuEpo for shorter periods of time. However, large chronic doses of rHuEpo have been shown to induce thrombocytopenia which could be explained by competition of erythroid and megakaryocytic stem cells seeing as they share a common progenitor.

Hypoxia or lack of oxygen, as a result of anemia may also be responsible for increases in platelet counts. It has been documented in other species as well as humans, that short-term exposure to hypoxia can cause an increase in platelet numbers. Studies in mice determined that hypoxia is sufficient to induce platelet production as early as 1-3 days later. One hypothesis to explain this phenomenon is an increased release of platelets from megakaryocytes. Seeing as anti-D can cause anemia which could lead to hypoxia it is possible anti-D increases platelet counts in ITP patients by inducing increased production of erythropoietin.

With respect to this topic, previous work on the murine glycoporphin A antibody TER-119 has demonstrated that this antibody is able to ameliorate ITP yet can cause anemia in mice independently of FcR or complement. TER-119 has been studied by various research groups that have consistently been able to demonstrate that TER119 can protect against passive ITP in mice. Therefore it could be possible that the anemia induced by TER119 could be causing a downstream signaling pathway which may be contributing to the therapeutic efficacy of this antibody and possibly other anti-erythrocyte antibodies.
Chapter 4

4 RATIONALE AND HYPOTHESIS

4.1 Brief Background

Polyclonal anti-D is in limited supply and its widespread use in ITP patients is in part hampered by the priority given to the prevention of alloimmunization in pregnant D- women\(^{50}\). In addition, its administration can be accompanied by adverse effects such as acute hemolysis and in extreme cases, renal failure. Because anti-D is a donor-derived product that carries theoretical risks of infection, hemolysis, and is in limited supply, it would be desirable to have a donor free alternative product that could treat ITP with minimal side effects.

Monoclonal antibodies to RBCs are newly emerging as potential therapies in ITP and are very useful tools to elucidate the mechanisms involved with anti-D administration\(^{71-74}\). Using a murine model of passive ITP, previous work in our laboratory has shown that some but not all erythrocyte-specific monoclonal IgG antibodies can increase platelet counts\(^{71}\) (Also refer to; Murine Model of Autoimmune Hemolytic Anemia: Red Blood Cell Clearance Mechanisms and Treatment Efficacy by Xi Chen, MSc thesis, November 2012). We have also observed a positive correlation between the amelioration of ITP and a decrease in RBC counts by therapeutic anti-erythrocyte antibodies in thrombocytopenic mice\(^{71,92}\). It isn’t fully understood how erythrocytes are being cleared and whether or not the clearance is required for the ameliorative effect of these antibodies in ITP. A better understanding of the role of RBC clearance in the therapeutic effect of these erythrocyte-specific antibodies could help determine whether this side effect could be removed from a future therapy.

4.2 Rationale

The MPS blockade theory of anti-D action hypothesizes that opsonized red blood cells reduce platelet destruction by competitive inhibition of Fc\(\gamma\)R-dependent clearance. If this is true, anti-
RBC antibodies that cause RBC clearance* in vivo should also cause a reduction of phagocytosis of RBCs in vitro. In addition, antibodies which cause RBC clearance and ameliorate ITP in vivo should prevent platelet phagocytosis in an in vitro model when both opsonized RBCs and platelets are added together.

In this work, in vitro phagocytosis experiments were performed in order to clarify whether it is the competitive inhibition of phagocytosis by the presence of opsonized RBCs that increases platelet counts or some other mechanism that has not yet been explored. A variety of monoclonal anti-erythrocyte antibodies of different antigen specificities, antibody isotypes and ability to ameliorate ITP (Table 1) were used in vitro to clarify the mechanism by which RBC clearance is occurring following treatment with anti-RBC antibodies.

The use of monoclonal antibodies would allow further study into the mechanism of anti-D in ITP to determine whether or not adverse events, such as erythrocyte clearance, are important in anti-D’s action or whether this characteristic of anti-RBC antibodies could be considered dispensable.

4.3 Hypotheses

1. Anti-erythrocyte antibodies that cause RBC clearance in vivo will cause RBC phagocytosis in vitro.

* It should be noted that throughout these experiments, we are measuring changes in the concentration of RBCs in the blood of treated mice. For the purpose of this thesis, a significant decrease in the concentration of RBCs following anti-RBC antibody injection will be referred to as RBC clearance.
1. Mouse glycophorin A
2. Hen Egg Lysosome
3. Human Duffy Protein
4. Ovalbumin
5. Human glycophorin A

5 MATERIALS AND METHODS

5.1 MICE

C57BL/6 (B6) mice (6-10 weeks old) were purchased from Charles River Laboratories (St-
Constant, PQ, Canada) and housed in the St. Michael’s Hospital Research Vivarium until used.
A unique line of transgenic mice known as HOD mice were recently made available to us from
the laboratory of Dr. Jim Zimring (Emory University, Atlanta). These transgenic mice have
erthrocytes that express an antigen consisting of human Duffy multipass transmembrane protein
in tandem sequence with ovalbumin (OVA) and hen egg lysozyme (HEL) on the extracellular
face of the erythrocyte-plasma membrane (Figure 2). HOD mice on the FVB background were
created by the Zimring group as described. Human GPA mice were a gift from Dr. Narla
Mohandas from the New York blood center. Both HOD and hGPA mice were housed and bred
in the St. Michael’s Hospital Research Vivarium. This study used both female and male HOD

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<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Reactivity</th>
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<tr>
<td>TER119</td>
<td>Rat IgG2b</td>
<td>mGPA1</td>
</tr>
<tr>
<td>Deglycosylated TER119</td>
<td>Rat IgG2b</td>
<td>mGPA1</td>
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<tr>
<td>34-3C</td>
<td>Mouse IgG2a</td>
<td>Band3</td>
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<td>6A7</td>
<td>Mouse IgG1</td>
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mice (5-10 weeks old). All mice were kept in a controlled environment at the St. Michael’s Hospital Research Vivarium and all animal experiments were approved by the Animal Care and Use Committee of St. Michael’s Hospital.

**Figure 2. Hen egg lysozyme-Ovalbumin-Duffy (HOD) erythrocyte model.**
Hen egg lysozyme-Ovalbumin-Duffy (HOD) mice express the HOD antigen on the surface of erythrocytes which consist of a Duffy multipass transmembrane protein in tandem sequence with ovalbumin and hen egg lysozyme. HOD mice were generated and supplied to us on the FVB background. Taken from reference\(^9\)

### 5.2 Reagents

**Phosphate buffered saline** (PBS, pH: 7.22) supplemented with 10% citrate-phosphate-dextrose solution with adenine (CPDA) (PBS-CPDA) was used to incubate with whole blood for the purpose of counting platelets and erythrocytes during in vivo experiments. **Buffered Saline**

**Glucose Citrate Buffer** (see Table 2 for recipe). **Anticoagulant Buffer** (see Table 3 for recipe).

**Hank’s Buffered Saline Solution** purchased from Invitrogen. **Anti-platelet antibodies:**

Monoclonal anti–integrin αIIb antibody (MWReg30, rat, IgG1) was purchased from BD Biosciences (Mississauga, ON, Canada) and Nit G (anti-GPIb antibody) was kindly provided by Dr. Heyu Ni (St Michael’s Hospital, Toronto, ON, Canada). **Secondary antibodies:** R-Phycoerythrin (PE) goat anti-rat IgG was purchased from BD Biosciences. R-PE goat anti-mouse IgG was purchased from Jackson Immuno Research Ltd. R-PE donkey anti-rabbit IgG was purchased from e Bioscience (ebioscience.com). **IgG control:** Chrompure rat and mouse IgG fraction (whole molecule) were both purchased from Jackson Immuno Research. **Erythrocyte antibodies:** monoclonal anti-GPA antibody (TER-119, rat, IgG2b) was purchased from BD
Bioscience and BioXcell (West Lebanon, NH, USA) and monoclonal anti Band-3 antibody (34-3C, mouse, IgG2a) was purchased from Hycult Biotech. M1/69, anti CD24 rat IgG2b was purchased from Affymetrix eBioscience. **HOD specific antibodies:** Monoclonal anti-HEL antibodies (5B9, 4B7, both mouse IgG1 antibodies) were a special gift from the laboratory of Dr. James C Zimring (Emory University, Atlanta) and manufactured and purified by BioXcell. Polyclonal rabbit anti-OVA IgG was purchased from Polyscience Inc. Monoclonal anti-Duffy Fy3 antibody (MIMA-29, mouse, IgG2a) was kindly provided by Dr. Gregory Halverson from the New York Blood Center. Monoclonal anti-Duffy Fy3 antibody (CBC-512, mouse, IgG1) was a kind gift from Dr. Makoto Uchikawa (Japanese Red Cross Central Blood Centre).
Table 2. Buffered Saline Glucose Citrate (BSGC) Recipe

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
<th>Gr/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisodium Citrate Dihydrate (Mw= 294.1 g/mol)</td>
<td>13.6mM (0.0136M)</td>
<td>4 gr/L</td>
</tr>
<tr>
<td>NaCl(Mw= 58.44)</td>
<td>116 mM(0.116M)</td>
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<tr>
<td>Na₂HPO₄ x 7H₂O(Mw= 268.07)</td>
<td>8.6mM (0.0086 M)</td>
<td>2.31 gr/L</td>
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<tr>
<td>KH₂PO₄(Mw=136.1)</td>
<td>1.6 mM (0.0016 M)</td>
<td>0.22 gr/L</td>
</tr>
<tr>
<td>Disodium EDTA Dihydrate (Mw= 372.24)</td>
<td>0.9 mM (0.0009 M)</td>
<td>0.335 gr/L</td>
</tr>
<tr>
<td>Glucose (Mw= 180.16)</td>
<td>11.1 mM(0.0111M)</td>
<td>2 gr/L</td>
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<tr>
<td>Carbacyclin (MW= 350.49)</td>
<td>1 ug/mL- add fresh everytime</td>
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</table>

Table 3. Anticoagulant Buffer recipe

<table>
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<th>Ingredient</th>
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<tbody>
<tr>
<td>Trisodium Citrate Dihydrate (Mw= 294.1 g/mol)</td>
<td>130 mM (0.13M)</td>
<td>38.23 gr/L</td>
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<tr>
<td>Disodium EDTA Dihydrate (Mw= 372.24)</td>
<td>10 mM (0.01M)</td>
<td>3.722 gr/L</td>
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<tr>
<td>Theophyllin (Mw= 180.16)</td>
<td>10 mM(0.01M)</td>
<td>1.8016 gr/L</td>
</tr>
<tr>
<td>Carbacyclin (MW= 350.49)</td>
<td>2 ug/mL- add fresh everytime</td>
<td></td>
</tr>
</tbody>
</table>

5.3 Platelet count

Ten µL of blood was acquired from each mouse from the saphenous vein. The blood was diluted in a solution of PBS-CPDA at 1:100. Each sample was centrifuged at 170g for 2 min in room temperature and platelet rich plasma (PRP) was collected. Two hundred µL of PRP was diluted 1:50 in a filtered isoton solution and the concentration of platelets was measured using a Beckman Z2 Coulter Counter (Beckman Coulter).
5.4 Erythrocyte count

Ten µL of blood was acquired from each mouse from the saphenous vein. The blood was diluted in a solution of PBS-CPDA at 1:100. The samples were diluted a further 1:1000 in PBS and the concentration of erythrocytes was measured by flow cytometry (Guava easyCyte flow cytometry system).

5.5 Antibody injections

Antibodies were injected into mice using the intravenous (IV) route by injecting the tail vein with a 27 gauge needle. The antibodies used for injection were previously prepared or diluted in PBS to a desired concentration and 200µL of the preparation was injected.

5.6 Induction of passive immune thrombocytopenia (ITP)

ITP was induced in CD1, HOD and hGPA mice by IV injection of the anti-platelet antibody (MWReg30). Three µg/mouse of MWReg30 was administered unless otherwise stated. This dose was chosen based on its ability to induce an optimal level of thrombocytopenia observable at 24 hours after injection. This was evaluated by a dose response analysis.

5.7 Treatment of ITP with anti-erythrocyte antibodies

Please refer to Table 1 for a complete list of erythrocyte-specific antibodies used in this thesis. Erythrocyte-specific antibodies (5B9 and 4B7) reactive with the HEL portion of the HOD molecule on erythrocytes were used in the treatment of murine ITP in HOD mice. These antibody doses (5B9 (60 µg) and 4B7 (60 µg) were selected based upon independent dose response studies on HOD mice (Supplemental Figure 2).

Rabbit polyclonal antibody specific for OVA bound to HOD erythrocytes (Appendix; Supplemental Figure 2) and was used to treat murine passive ITP in HOD mice. Anti-OVA was used at a dosage of 120 µg/mouse based upon a dose response analysis (Supplemental Figure 1).

Erythrocyte-specific monoclonal antibodies reactive with the Duffy portion of the molecule (MIMA-29 (60 µg), CBC-512 (8 µg)) were also used in HOD mice to treat murine passive ITP. The dosages used in the treatment of ITP were based upon independent dose response studies. The Glycophorin A specific monoclonal antibody which reacts with all murine erythrocytes...
(TER-119) was also used in the treatment of HOD mice (30 µg/mouse) as well as CD-1 mice (40 µg/mouse). The band-3 specific monoclonal antibody which reacts with all murine erythrocytes (34-3C) was previously shown to ameliorate ITP. The CD-24 specific monoclonal antibody (M1/69) was previously shown to ameliorate ITP.

Mice were injected intravenously with the above erythrocyte-specific antibodies, returned to their cages for the times indicated and then injected with antiplatelet antibody (MWReg30) to induce thrombocytopenia. The mice were again returned to their cages and then bled for platelet and erythrocyte enumeration at the time points indicated.

5.8 Erythrocyte phagocytosis assay

5.8.1 Preparation of RAW264.7 cells

Cells were harvested by scraping into fresh RPMI/FBS 10%, obtained cell count using Beckman Coulter Vi-Cell XR, Cell viability Analyzer (Serial N° AT08066) and adjusted to 0.5x10⁶ cells/mL. Cells were cultured at 1 mL/well of the previous preparation in 12 well plates with coverslips. Cells were incubated overnight at 37°C.

5.8.2 Erythrocyte enumeration

Five to eight hundred µL of whole blood was acquired from each mouse using the cardiac puncture and the blood immediately diluted in 200 µl of a 1:1 (Anticoagulation buffer: BSGC buffer) and further diluted to 1.5 mL using BSCG buffer. Each diluted blood sample was centrifuged at 300g for 3 min in room temperature and platelet rich plasma (PRP) was removed. RBCs were then resuspended in 1 mL PBS and each sample was diluted 1:3000 in PBS and then analyzed by flow cytometry (Guava EasyCyte flow cytometry system) to determine the concentration of erythrocytes in the blood.

5.8.3 Opsonization of RBCs with anti-RBC antibodies

Red blood cells were counted using the Guava EasyCyte Mini (Serial N° 2800060170) and adjusted to 5 x 10⁸ RBCs/mL. One mL of RBCs was used per antibody. Antibodies to RBC suspensions were added at the indicated concentrations and the mixture incubated for 1 hr at 37°C with gentle mixing.
5.8.4 Incubation with RAW264.7 cells

Following 1 hr incubation with antibodies, RBCs were washed 2 times with PBS 1X and counted again on the guava and adjusted to 0.2x10^8 RBCs/mL in RPMI-1640 supplemented with 10% heat-inactivated FBS. Media was removed from cells and 0.5 mL (10x10^6 RBCs; ratio ~1:20) of mouse RBCs were added per well. Cells were incubated at 37°C for 30 min.

5.8.5 Fixation of Cells

Phagocytosis was stopped by placing the cells on ice. Cells were washed once with ice cold PBS. Bound RBCs were lysed by adding 0.9 mL of dH2O for 1.5 min followed by 0.1 mL PBS 10X. Cells were washed one more time with ice cold PBS 1X. Cells were fixed by adding 0.5 mL of Fixation Buffer (4% Paraformaldehyde) and incubated for 20 min at 4°C.

5.8.6 Calculation of the Phagocytic Index

Pictures were taken using the Nikon Eclipse TS100 inverted microscope. Five pictures were taken per well (top, center, bottom, left and right) and internalized RBCs and macrophages were counted using Fiji (Fiji is Just Images) cell counting program. The Phagocytic Index (PI) was calculated using the following equation:

\[
PI = \left( \frac{\text{Total number of engulfed RBCs}}{\text{Total number of counted macrophages}} \right) \times 100
\]

5.9 Immunofluorescence detection of opsonized erythrocytes

Five to eight hundred µL of whole blood was acquired from each mouse using cardiac puncture and the blood immediately diluted in 200 µL of a 1:1 (Anticoagulation buffer: BSGC buffer) and further diluted to a total volume of 1.5 mL using BSCG buffer. Each diluted blood sample was centrifuged at 300g for 3 min in room temperature and platelet rich plasma (PRP) was removed. RBCs were then resuspended in 1 mL PBS and each sample was diluted 1:3000 in PBS. RBCs were counted using the Guava EasyCyte Mini (Serial N° 2800060170) and adjusted to 5 x 10^8 RBCs/mL. One mL of RBCs was used per antibody. Antibodies were added to RBC suspensions at the indicated concentrations. The mixtures were incubated for 1 hr at 37oC with gentle mixing. Following incubation RBCs were washed and readjusted to 10^8 RBCs/mL, 100 µL of the sample was added to 5 mL flow cytometry tubes and incubated in 100 µL of a preparation of the
appropriate species specific R-PE-conjugated secondary antibody (1:200) for 30 min at room temperature. A final wash was performed to remove the unbound antibody. The samples were then analyzed by flow cytometry (Guava EasyCyte flow cytometry system) to determine the mean fluorescence intensity (MFI) of the antibody-opsonized erythrocytes.

5.10 Erythrocyte to macrophage binding assay

The binding assay was conducted under the same conditions as the erythrocyte phagocytosis assay except everything was done on ice to prevent macrophages from ingesting RBCs. In addition, bound RBCs were not lysed with water as to allow counting of bound RBCs. Binding efficiency was calculated as follows:

\[
Binding\ efficiency = \left( \frac{Total\ number\ of\ surface\ bound\ RBCs}{Total\ number\ of\ counted\ macrophages} \right) \times 100
\]

5.11 Active inhibition assay

The inhibition assay was conducted in the exact same conditions as the erythrocyte phagocytosis assay. In this assay RBCs were incubated with either MIMA29 5 µg/mL or TER-119 at 5 µg/mL. In order to determine whether MIMA29 was able to inhibit phagocytosis, it was first added to RAW cells for 30 minutes at 37°C and 5% CO₂. Following incubation MIMA29 cells were removed and lysed using H₂O for 2 minutes. Then TER119 opsonized cells were added. There was a control well which contained both MIMA29 and TER119 opsonized RBCs at the same time to determine if it was simply a competition event. Following incubation, RBCs were washed away, lysed and RAW macrophages were fixed using 4% PFA.

5.12 Platelet phagocytosis assay

5.12.1 Preparation of RAW264.7 cells

Raw cells were harvested by scraping into fresh RPMI-1640 supplemented with 10% heat-inactivated FBS, and cells were enumerated using a Beckman Coulter Vi-Cell XR, Cell viability Analyzer (Serial No. AT08066) and adjusted to 5x10⁵ cells/mL. Cells were cultured in 12 well plates with coverslips using 1 mL of the cell preparation per well. Cells were incubated overnight at 37°C.
5.12.2 Platelet and Erythrocyte enumeration

Five to eight hundred µL of whole blood was acquired from each mouse using cardiac puncture. Blood was immediately mixed with 200 µL of a 1:1 (Anticoagulation buffer: BSGC buffer) and diluted to a final volume of 1.5 mL using BSCG buffer. Each diluted blood sample was centrifuged at 300g for 3 min at room temperature and platelet rich plasma (PRP) was collected. Remaining sample was again resuspended to 1.5 mL in BSGC and centrifuged again. PRP was again collected and added to the previous PRP sample, this PRP mixture was then centrifuged at 1200 g for 10 min. The platelet pellet was resuspended in 1 mL BSGC and 5 µL of PRP was diluted 1:200 in BSGC buffer then platelets were counted on a MACSQuant analyzer 10 (MACS Miltenyi Biotec) flow cytometer to determine the concentration of platelets in the preparation.

RBCs were then resuspended in 1 mL PBS and each sample was diluted 1:3000 in PBS and then analyzed by flow cytometry (Guava EasyCyte flow cytometry system) to determine the concentration of erythrocytes in the blood.

5.12.3 Labeling of platelets with CMFDA Cell Tracker Green

Platelets were counted using the MACSQuant analyzer 10 (MACS Miltenyi Biotec) flow cytometer by taking 5 µL of PRP and diluting in 995 µL of BSGC buffer. Platelets were adjusted to 5 x 10⁸ platelets/mL. CMFDA was prepared at a concentration of 10 µg/mL. Then equal volumes of platelets and CMFDA (e.g. 1 mL of platelet and 1 mL of CMFDA) were mixed together for a final CMFDA concentration of 5 µg/mL. Mixture was incubated for 30 mins at 37°C with gentle mixing in the dark.

5.12.4 Opsonization of platelets with anti-CD41 (Mwreg30) antibody and of RBCs with anti-RBC antibodies

Following incubation with CMFDA platelets were centrifuged at 1200 g for 10 min and pellet was resuspended in 1 mL of HBSS. Mwreg30 antibody was added to platelet samples at a concentration of 10 µg/mL. Mixture was incubated for 30 mins at room temperature with gentle mixing.

RBCs were counted using the Guava Easy Cyte Mini (Serial No. 2800060170) and adjusted to 5 x 10⁸ RBCs/mL. Anti-RBC antibodies of select concentrations, were added to one mL of RBCs. Mixture was incubated for 1 hr at room temperature with gentle mixing.
5.12.5 Incubation with RAW264.7 cells

Following 1 hour incubation with antibodies, RBCs were washed with PBS and centrifuged for 300 g for 8 mins. RBCs were counted again and adjusted to $0.4 \times 10^8$ RBCs/mL using RPMI-1640 supplemented with 10% heat-inactivated FBS. Platelets were washed with HBSS and centrifuged at 1200g for 10 mins. Platelets were counted again and adjusted to $3-5 \times 10^8$ platelets/mL into RPMI-1640 supplemented with 10% heat-inactivated FBS. To add the RBCs and platelets, supernatant was removed from RAW 264.7 cells and 100 µl of the platelet preparation was added (3-5 x10^7 platelets; ratio 1 macrophage: 100 platelets) and 250 µl of the RBC preparation was added (10x106 RBCs; ratio ~1 macrophage: 20 platelets) per well. Cells were incubated at 37°C for 30 min.

5.12.6 Post phagocytosis preparation

Phagocytosis was stopped by placing the cells on ice. RAW 264.7 cells were washed once with 500 µL HBSS (1 µg/mL carbacyclin) per well. Remaining RBCs were lysed by adding 0.9 mL of dH$_2$O per well for 1.5 mins then 0.1 mL PBS 10X was added to stop the lysing process. Cells were washed 2 more times with 500 µL HBSS. Finally, 500 µL of a solution of PBS/0.5 mM EDTA /0.05% trypsin was added to wells for 5 mins at 37 degrees to remove any remaining bound platelets. The trypsin/EDTA solution was removed and cells were place in 500 µL RPMI 1640 containing HEPES buffer.

5.12.7 Confocal imaging and calculation of the Phagocytic Index

Pictures were taken using the LSM 700 Zeiss Confocal microscope. Five pictures were taken per well (top, bottom, center, left and right). Internalized platelets were counted using IMARIS 8.0. The criteria of an internalized platelets in these experiments were: a minimum volume of 4.2 µM, green florescence and internalization of the platelet by the macrophage on the x, y and z planes. Macrophages were counted using Fiji (Fiji is Just Images) cell counting program. The Phagocytic Index (PI) was calculated using the following equation:

$$ PI = \left( \frac{\text{Total number of engulfed platelets}}{\text{Total number of counted macrophages}} \right) \times 100 $$
Chapter 5

6 RESULTS

6.1 Erythrocyte-specific antibodies in RBC clearance and amelioration of murine ITP

Previous work done by Mark Jen and Xi Chen in our lab have examined the ability of various antibodies to bind to RBCs, cause RBC clearance and ameliorate ITP (Table 4). Part of this thesis work was to contribute to this effort and evaluate two additional antibodies that have not been tested for their in vivo efficacy, these antibodies include a polyclonal anti-OVA (anti-OVA) which targets the OVA portion of the HOD molecule (Figure 2) and a monoclonal antibody (6A7) which targets the human glycophorin A antigen. The HOD molecule is located on the surface of red blood cells in HOD mice and 6A7 is located on the surface of RBCs of transgenic hGPA mice.

Rabbit polyclonal anti-OVA was chosen because rabbit IgG is considered to bind well to murine FcRs to initiate phagocytosis and antibodies specific to the OVA portion of the molecule had not yet been tested for their ability to ameliorate ITP. OVA is important to target because antibodies specific to the HEL portion of the molecule showed low binding to HOD RBCs whereas antibodies to the Duffy portion bound well but didn’t cause any amelioration in ITP. Therefore evaluating the ability of OVA specific antibodies to ameliorate ITP in HOD mice would create a more complete analysis.

The human glycophorin A specific antibody 6A7 was selected because it is important to target multiple antigens when trying to establish a therapeutic role for anti-RBC antibodies in ITP. Although there exist a variety of antibodies reactive with the HOD antigen which have been evaluated for their ability to ameliorate ITP, they only allow for analysis of anti-inflammatory activity using one antigen. In addition, this antibody targets a human antigen which could make it easier to transition to clinical trials if the antibody is found to be effective in increasing platelet counts.

To further extend the impact of our studies, an anti-CD24 antibody (M1/69), purchased from eBioscience was evaluated to determine if this batch of antibody was able to ameliorate ITP.
Although M1/69 has previously been shown to ameliorate ITP\textsuperscript{71} it was later demonstrated in our lab to be ineffective (refer to: Murine Model of Autoimmune Hemolytic Anemia: Red Blood Cell Clearance Mechanisms and Treatment Efficacy by Xi Chen, MSc thesis, November 2012). Therefore we needed to evaluate the ability of this specific antibody to ameliorate ITP before using it in the phagocytosis assays.

<table>
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<th>Antibody</th>
<th>Isotype</th>
<th>Reactivity</th>
<th>RBC binding$^1$</th>
<th>RBC clearance$^2$</th>
<th>Ameliorate ITP?</th>
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<td>TER119</td>
<td>RatIgG2b</td>
<td>mGPA</td>
<td>++++</td>
<td>++++</td>
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<td>34-3C</td>
<td>Mouse IgG2a</td>
<td>Band3</td>
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<td>++++</td>
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</tr>
<tr>
<td>M1/69</td>
<td>Rat IgG2b</td>
<td>CD24</td>
<td>++++</td>
<td>++++</td>
<td>Yes/No</td>
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<tr>
<td>4B7.B1</td>
<td>Mouse IgG1</td>
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<td>HEL</td>
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<td>-</td>
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<td>Mouse IgG1</td>
<td>Fy3</td>
<td>+++</td>
<td>++++</td>
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</table>

6. RBC binding denotes how well the antibody bound to RBCs which was measured by flow cytometry ($++++ = $ MFI > 500; $+++ = $ MFI > 250; $++ = $ MFI > 125; $+ = $ MFI > 62.5; $- = $ MFI < 62.5)

7. RBC clearance denotes how well an antibody was able to decrease the concentration of RBCs in the blood ($++++ = > 50\%$ clearance; $+++ = > 25\%$; $++ = > 12.5\%$; $+ = > 6.25\%$; $- = < 6.25\%$)

### 6.1.1 Anti-OVA polyclonal antibody

The first antibody tested was a commercially obtained polyclonal rabbit anti-OVA. This work was done in collaboration with Mark Jen (MSc student, Lazarus laboratory).

The antibody was first tested for its ability to bind to HOD RBCs. In vitro flow cytometric analysis showed that this antibody bound to erythrocytes from HOD mice (Figure 3). According to a dose response curve, in vivo flow cytometric analysis with HOD erythrocytes observed a reduction in erythrocyte binding, although the cells were still positive for the antibody 24 hours post injection (Supplemental Figure 1). When examined for anti-OVA’s ability to induce RBC clearance, we observed that this antibody could not induce RBC clearance in HOD mice at any doses examined up to 120 µg/mouse (Figure 4). Anti-OVA antibody was then examined for its ability to ameliorate ITP. The result showed this antibody, like the anti-HEL and anti-Duffy antibodies, did not ameliorate ITP in HOD mice (Figure 5).
Figure 3. In vitro flow cytometric analysis of HOD erythrocytes with HOD specific antibodies. Whole blood was acquired from HOD mice and enumerated for erythrocyte concentration. Erythrocytes (10⁷ cells) were incubated with all the depicted primary antibodies at 5 µg/mL then followed by a species specific R-PE conjugated secondary antibody at 1:200. Antibody-opsonized erythrocytes were analyzed by flow cytometry to determine the mean fluorescence intensity (MFI). All the anti-HEL antibodies when compared to the anti-Duffy antibodies have relatively lower MFI. The anti-OVA polyclonal antibody appeared to react to a similar degree as compared to anti-HEL antibodies but a different secondary antibody was used complicating a direct comparison. (n= 3 per group) Figure from Mark Jen’s MSc thesis⁹².
Figure 4. Effects of anti-OVA IgG on erythrocyte clearance in HOD mice.

Left Panel. Mice were uninjected (■—■) or injected with 30 µg of antibody TER119 (●—●), 60 µg of rabbit IgG (▲—▲), or 120 µg of anti-OVA IgG (Δ—Δ) then bled at the time points shown; Erythrocytes were enumerated at each time point. No significant anemia was observed for mice injected with anti-OVA antibody. (n=3 per group)

Right Panel. The erythrocytes acquired at each time point were reactive with a species specific R-PE conjugated secondary antibody (R-PE goat anti-rat IgG for TER119; R-PE donkey anti-rabbit IgG for anti-OVA). Sample were then analyzed by flow cytometry to determine the MFI of the antibody-opsonized erythrocytes. The data from the figure on the right corresponds to the same bleeds as the figure on the left. The MFI for TER119 was excluded for an easier comparison. (n=3 per group)
Figure 5. Effects of anti-OVA IgG on the amelioration of passive ITP in HOD mice. HOD mice were untreated or treated with PBS, antibody TER119, or anti-OVA antibody at the doses shown and then ITP induced by anti-platelet antibody (MWReg30). Platelets were enumerated 24 hours later. Only mice treated with TER119 had significantly higher platelet counts than mice given only PBS (P<0.05). (n=3 per group)
6.1.2 Anti-hGPA antibody 6A7

We have also acquired a strain of hGPA mice commonly referred to as the human Glycophorin A expressing mice which express hGPA on the surface of mouse RBCs. These mice were used to examine the ability of an hGPA specific antibody (6A7) to bind RBCs, cause RBC and platelet clearance and ameliorate ITP.

First, the ability of this antibody to bind to hGPA erythrocytes was assessed followed by its ability to clear RBCs and platelets. In comparison to control IgG, the antibody reacted with erythrocytes from hGPA mice in vivo (Figure 6C). Second, was to test the antibody’s ability to induce anemia and to treat murine passive ITP. Having previously established that TER-119 can induce anemia and ameliorate ITP in B6 mice, TER-119 was used as a positive control for all the experiments conducted. This antibody did not cause anemia (Figure 6A) nor did it cause thrombocytopenia (Figure 6B). Once these criteria were established we moved on to testing the therapeutic ability of the antibody.

As demonstrated in Figure 7, the antibody 6A7 was not able to ameliorate ITP 24 hours after injection; however, it was observed that at 24 hours post injection the antibody was no longer present in the mice (Figure 6C). Therefore we evaluated the ability of 6A7 to ameliorate ITP while it was still present in the mouse (i.e. at 2 hours post injection). Inducing ITP at this earlier time still didn’t improve platelet loss by the antiplatelet antibody. Therefore we believe 6A7 is not able to ameliorate ITP.
Figure 6. 6A7 RBC and platelet clearance in hGPA mice.

Mice were uninjected (○—○), injected with 60 µg Mouse IgG (□—□), 45 µg of antibody TER-119 (▽—▽) or 60 µg of 6A7 (△—△). A. Mice were bled and erythrocytes enumerated at the time points shown. (n=1 per dose) left panel. RCB clearance. B. Platelets were enumerated at the time points shown. C. Erythrocytes from the same mice were evaluated for the relative level of in vivo 6A7 antibody binding by reacting the erythrocytes with a goat anti-mouse R-PE conjugated secondary antibody. D. Antigen Loss was measured by re opsonizing RBCs 24 hrs post injection and remeasuring the MFI. (n=4 per group)
Figure 7. Effects of antibody (6A7) on the amelioration of passive ITP in hGPA mice.

**Left panel.** Mice were untreated or treated with PBS, antibody TER-119 or 6A7 at the doses shown for 24 hours then ITP induced by injecting the mice with anti-platelet antibody (MWRReg30). Platelets were enumerated 24 hours later. (n= 3-4 per group)**

**Right panel.** Mice were untreated or treated with PBS, TER-119, or 6A7 at the doses shown for 2 hours before ITP was induced. Mice treated with TER-119 had significantly higher platelet counts than mice given PBS but not the 6A7 treated group (P<0.05). (n= 6 per group)
6.1.3 Anti-CD24 antibody

These experiments were carried out by Andrew Crow. Based on the results in Figure 8, this antibody does in fact ameliorate ITP in a passive mouse model. In addition, RBC counts for mice injected with M1/69, although decreased, did not reach significance in this small series of experiments.

**Figure 8. Effects of antibody (M1/69) on the amelioration of passive ITP in C57BL6 mice.**

**Left panel.** Mice were untreated or treated with antibody TER119 (45µg) or M1/69 (50 µg) for 30 minutes then ITP was induced by injecting the mice with 2µg anti-platelet antibody (MWReg30) per mouse. RBCs were enumerated 24 hours later. (n= 6 per group) **Right panel.** Mice were untreated or treated with antibody 45 µg TER-119 or 50 µg M1/69 for 30 minutes then ITP was induced by injecting the mice with 2µg anti-platelet antibody (MWReg30) per mouse. Mice treated with TER-119 and M1/69 had significantly higher platelet counts than untreated mice (n= 6 per group). The experiments in this figure were performed by Andrew Crow as a collaborative effort to help establish that the same batch of M1/69 used in these phagocytosis experiments do in fact ameliorate ITP. *** (P<0.05)

6.2 Erythrocyte-specific antibodies and mediation of RBC phagocytosis in vitro

Anti-erythrocyte antibodies previously used vary in their isotype, reactivity, ability to cause RBC clearance and ameliorate ITP (Table 5). However, it isn’t clear why some antibodies are able to cause RBC clearance while others are not and whether this clearance is involved in the therapeutic effect. Work done by Mark Jen in our lab suggests that anemia may not be a strict requirement for the amelioration of ITP seeing as TER119 ameliorative effects occur before anemia has been established92. However, the role and mechanism of RBC clearance in the effectiveness of anti-RBC antibodies in ITP has not yet been established. The MPS blockade theory suggests that anti-RBC antibodies ameliorate ITP by inhibiting the ability of platelets to bind Fc receptors on macrophages and being phagocytosed due to competition with opsonized
RBCs. To further investigate this phenomenon, several in vitro experiments have been carried out to determine whether RBC clearance by these anti-RBC antibodies is related to FcγR mediated phagocytosis or potentially to another non-antibody mediated mechanism of clearance. Using RAW 264.7 macrophages we have evaluated the ability of the following anti-erythrocyte antibodies (Table 5) to induce phagocytosis of opsonized RBCs.

**Table 5. Anti-erythrocyte antibodies used in RBC phagocytosis**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Reactivity</th>
<th>RBC binding¹</th>
<th>RBC clearance²</th>
<th>Ameliorate ITP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TER119</td>
<td>RatIgG2b</td>
<td>mGPA</td>
<td>++++</td>
<td>++++</td>
<td>Yes</td>
</tr>
<tr>
<td>Deglycosylated TER119</td>
<td>RatIgG2b</td>
<td>mGPA</td>
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<td>-</td>
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<tr>
<td>M1/69</td>
<td>Rat IgG2b</td>
<td>CD24</td>
<td>+++</td>
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</tbody>
</table>

1. RBC binding denotes how well the antibody bound to RBCs which was measured by flow cytometry (++++ = MFI > 500; +++ = MFI > 250; ++ = MFI > 125; + = MFI > 62.5; - = MFI < 62.5)

2. RBC clearance denotes how well an antibody was able to clear RBCs from circulation (++++ = > 50% clearance; +++ = > 25%; ++ = > 12.5%; + = > 6.25%; - = < 6.25%)

6.2.1 RBCs sensitized with anti-mGPA antibody (TER119) are phagocytosed by RAW macrophages

Having previously established that TER119 can induce RBC clearance and ameliorate ITP in C57BL/6, HOD and CD-1 mice, TER119 was used in the phagocytosis experiments to determine whether it would be possible to study RBC phagocytosis using this protocol. Mouse RBCs were incubated with the antibody for 45 minutes at room temperature, washed and then added to RAW macrophages for 30 mins at 37 °C and 5 % CO₂. Following incubation, the remaining RBCs
were lysed with H$_2$O for 2 mins and RAW cells were fixed with 4% PFA before being visualized on a phase contrast microscope. Macrophages and internalized RBCs were counted and the phagocytic index was calculated. TER119 was able to opsonized RBCs for phagocytosis at concentrations as low as 1.25 µg/mL (Figure 9). The maximal RBC phagocytosis (i.e. Plateau) was achieved $\geq$ 5 µg/mL (Figure 9 & Figure 10). TER119, when deglycosylated did not induce RBC phagocytosis indicating that the function of the Fc regions is required for phagocytosis mediated by this antibody (Figure 11).

**Figure 9. Dose dependent phagocytic index of TER119 opsonized RBCs**
Erythrocytes were acquired from C57B/6 mice and non-opsonized (control) or opsonized with various concentrations of TER-119 then incubated with RAW264.7 macrophages for 30 minutes. Phagocytic index was calculated by counting the total number of ingested RBCs and diving this by the total number of macrophages in a field and multiplying by 100 (n= 5 per group). **$P$<0.01, ***$P$<0.001
Figure 10. **In vitro phagocytosis of TER119 opsonized erythrocytes.**

**Upper Panels.** Erythrocytes were acquired from a C57B/6 mouse and either non-opsonized (control) or opsonized with the indicated concentration of TER119 then incubated with RAW264.7 macrophages for 30 minutes. **Lower panel.** Higher resolution image of TER119 5 µg/mL group. The white arrow is indicating an empty macrophage and the red arrow indicates an internalized RBC.
RBCs collected by cardiac puncture from C57/B6 mice were opsonized with the indicated modification of TER119 and incubated with RAW264.7 macrophages in vitro. Phagocytic index was calculated. Control indicates opsonized RBCs were not phagocytosed (n= 4-5 per group) **P<0.05

6.2.2 RBCs sensitized with antibodies to the HOD molecule are not phagocytosed by RAW macrophages

The HOD specific antibodies which include: 4B7, 5B9, anti-OVA, CBC512 and MIMA29 were examined for their phagocytic abilities. All these experiments were carried out using RBCs from the HOD mice which express the HOD antigen on the surface of RBCs (Figure 2). The anti-HEL antibodies, 4B7 and 5B9, were the first to be examined. HOD RBCs opsonized with these antibodies at selected concentrations (5, 10, 20, and 40 µg) were unable to be phagocytosed as assessed by phase contrast microscopy (Figure 12).

The rabbit polyclonal anti-OVA antibody was tested next. This antibody targets the middle portion of the HOD antigen on the erythrocytes in HOD mice. Since the antibodies specific for the HEL antigen are of the IgG1 isotype it is possible that these antibodies have no effect on erythrocyte phagocytosis because of the isotype, the specific binding site, or other factors. When examined for anti-OVA’s ability to opsonized RBCs for phagocytosis, again we observed that this anti-OVA opsonized HOD RBCs were not phagocytosed at any concentrations up to 50 µg/mL (Figure 12). These antibodies also do not cause RBC clearance in vivo.92
Since HOD RBCs opsonized with HEL and OVA specific antibodies were not phagocytosed, antibodies to the Duffy portion of the HOD molecule were examined next. The first anti-Duffy antibody tested was MIMA-29 (mouse, IgG2a). MIMA-29 is able to cause RBC clearance in HOD mice with significant clearance at 4 and 6 days post-antibody injection\(^92\). Despite its ability to induce significant RBC clearance at a dose of 60 µg in mice, RBCs opsonized with this antibody were not phagocytosed at concentrations as high as 40 µg/mL (Figure 12). Next tested was CBC-512 (mouse, IgG1), an antibody kindly provided by Dr. Makoto Uchikawa of The Japanese Red Cross Central Blood Centre. Although the antibody was only available as a tissue culture supernatant, it is the same isotype (IgG1) as all of the anti-HEL antibodies used in this thesis. Mice injected with CBC-512 develop a rapid and severe anemia by 24 hours post injection. However, like MIMA-29, CBC512 opsonized RBCs were not phagocytosed (Figure 12).
Figure 12. Erythrocytes opsonized with HOD specific antibodies are not phagocytosed in vitro.

HOD erythrocytes were non-opsonized (control) or opsonized in vitro with indicated concentrations of 4B7 (A), 5B9 (B), CBC512 (C), MIMA29 (D) and anti-OVA(E), followed by incubation with RAW264.7 macrophages for 30 minutes. HOD antibodies are represented by filled circles (●) and bars represent the phagocytic index of TER119 (5 µg/ml) used as a positive control in each experiment. Phagocytic index was calculated using equation in the methods section. (n= 4-5 per group)
6.2.3 RBCs sensitized with the anti-hGPA antibody (6A7) are not phagocytosed by RAW macrophages

In order to target an antigen other than the HOD antigen, we also used a strain of transgenic human glycophorin A expressing mice which were kindly provided by Dr. Narla Mohandas (New York Blood Center). As mentioned earlier, this antibody (6A7) is unable to induce RBC clearance or ameliorate ITP in a mouse model (Figure 6A and Figure 7). We tested this antibody’s ability to opsonized RBCs for phagocytosis. Using the erythrocyte phagocytosis assay it was found that 6A7 opsonized RBCs were not phagocytosed even at concentrations of 40 µg/mL allowing us to conclude that this antibody does not allow for RBC phagocytosis (Figure 13).
Figure 13. Human glycophorin A specific anti-erythrocyte antibody’s inability to induce RBC phagocytosis in vitro.
Erythrocytes were from hGPA mice. Erythrocytes were opsonized with 6A7 (●) using 5, 10, 20 and 40 µg/mL. Bar represents the phagocytic index of TER119 (5 µg/ml) used as a positive control in these experiments. This antibody was unable to induce RBC phagocytosis. (n= 6 per group in 3 independent experiments)
6.2.4 RBCs sensitized with the anti-band-3 antibody (34-3C) are phagocytosed by RAW macrophages

It has previously been established that 34-3C can induce anemia and ameliorate ITP in C57BL/6 mice.\textsuperscript{90} We used this antibody in the phagocytosis experiments to determine whether erythrocytes opsonized with this antibody would be phagocytosed by RAW macrophages. The anti-Band 3 antibody, 34-3C was observed to opsonize RBCs for significant phagocytosis at concentrations at 5 µg/mL with a trend toward causing phagocytosis at concentrations as 2 µg/mL (Figure 14B).
Figure 14. Anti-band 3 antibody (34-3C) can induce RBC phagocytosis in vitro.
A. Erythrocytes were acquired from a C57B/6 mouse and either non-opsonized (control) or opsonized in vitro with the indicated concentrations of 34-3C followed by incubation with RAW264.7 macrophages for 30 minutes. B. Phagocytic index was calculated using equation in the methods section. (n= 4 per group) *P < 0.05, **P<0.01, ***P<0.001
6.2.5 RBCs sensitized with the anti-CD24 antibody (M1/69) are phagocytosed by RAW macrophages

The anti-CD24 antibody, M1/69, has been shown to both ameliorate ITP in a passive mouse model and cause RBC clearance in vivo. This antibody is a Rat IgG2b and targets the murine CD24 antigen which is found on erythrocytes, thymocytes, peripheral lymphocytes and myeloid lineage. This antibody bound well to erythrocytes and when it was used to opsonized erythrocytes, these erythrocytes were phagocytosed at concentrations starting at 5 µg/mL (Figure 15).
Figure 15. Anti-CD24 antibody can induce RBC phagocytosis in vitro.
A. Erythrocytes were acquired from a C57B/6 mouse and not opsonized (control) or opsonized in vitro with various concentrations of M1/69 then incubated with RAW264.7 macrophages for 30 minutes. B. Phagocytic index was calculated using equation in the methods section under erythrocyte phagocytosis assay. (n= 4 per group). ***P<0.001
### 6.3 Examination of the discrepancies of various antibodies to cause phagocytosis

There is a lack of erythrocyte phagocytosis observed when using the anti-Duffy antibodies, MIMA29 and CBC512, to opsonize RBCs despite the ability of these antibodies to induce RBC clearance in vivo. In order to address this inconsistency, four factors were examined. First was to assure the ratio of RBCs to macrophages was appropriate, second was the ability of the antibodies to bind to the RBCs, third was the ability of opsonized RBCs to bind to FcRs on macrophages and fourth was the ability of MIMA29 to interact with inhibitory FcRs to prevent phagocytosis of TER119 opsonized RBCs.

#### Table 6. Anti-erythrocyte antibodies ability to mediate RBC phagocytosis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Reactivity</th>
<th>RBC binding</th>
<th>RBC clearance</th>
<th>Ameliorate ITP</th>
<th>Causes Phagocytosis</th>
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<td>mGPA</td>
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<td>Yes</td>
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<td>Mouse IgG1</td>
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<tr>
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<td>Fy3</td>
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<td>Mouse IgG2a</td>
<td>Band3</td>
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<tr>
<td>M1/69</td>
<td>Rat IgG2b</td>
<td>CD24</td>
<td>++++</td>
<td>+++</td>
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<td>Yes</td>
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</table>

1. RBC binding denotes how well the antibody bound to RBCs which was measured by flow cytometry (++++ = MFI >500; +++ = MFI > 250; ++ = MFI > 125; + = MFI > 62.5; - = MFI < 62.5)

2. RBC clearance denotes how well an antibody was able to clear RBCs from circulation (+++ = > 50% clearance; +++ = > 25%; ++ = > 12.5%; + = >6.25%; - = < 6.25%)

#### 6.3.1 Effect of the erythrocyte to macrophage ratio on phagocytosis

The ratio of macrophages to RBCs used in all of the erythrocyte phagocytosis experiments in this thesis was 1:20 respectively. This ratio has been reported in several publications examining
phagocytosis\textsuperscript{97,98} however ratios as high as 1:100 have also been reported\textsuperscript{99}. To determine whether the lack of phagocytosis seen with MIMA29 and CBC512 was due to an insufficient ratio of macrophages to RBC, ratios of 1:20, 1:40 and 1:80 were used. Experiments were carried out using the same methods as with erythrocyte phagocytosis except in these experiments a higher ratio of RBCs was used. For 1:40 ratio the number of RBCs per well was increased to 2x10\textsuperscript{7} RBCs per well and for 1:80 ratio, 4x10\textsuperscript{7} RBCs were added to each well. Increasing the amount of RBCs did not result in the phagocytosis of MIMA29 or CBC512 opsonized RBCs and it actually decreased the phagocytosis of TER119 opsonized RBCs.

![Phagocytic Index (PI) vs. RBC:Macrophage ratio](image)

**Figure 16. Macrophage to erythrocyte ratio does not increase phagocytosis**

Erythrocytes were non-opsonized (○—○), opsonized with 5 µg/mL of TER119 (□—□), 40 µg/mL of MIMA29 (△—△) or 40 µg/mL of CBC512 (△—△) then incubated with RAW264.7 macrophages for 30 minutes and the phagocytic index was calculated. (n= 4 per group) *** (P<0.001)

### 6.3.2 HOD-specific antibody binding to HOD erythrocytes

To determine whether the lack of phagocytosis from the anti-Duffy antibodies was due to insufficient binding of the antibodies to RBCs, flow cytometry was used to examine antibody binding to erythrocytes. In order to examine antibody binding to red blood cells, a species specific R-PE conjugated secondary antibody was used and MFI was measured by a flow cytometer. Findings showed that MIMA-29 bound similarly to 34-3C, an antibody of the same isotype which can opsonize RBCs for phagocytosis (Figure 17). CBC512 bound less than MIMA29 and TER119 but more than the other HOD specific antibodies 4B7, 5B9 and polyclonal anti-OVA. However, the anti-HEL and anti-OVA antibodies bind much less
effectively and this could potentially explain why they can’t cause RBC clearance or phagocytosis.

![Graph showing MFI vs Concentration for different antibodies](image)

**Figure 17. HOD specific antibody’s abilities to bind HOD RBCs.**
Erythrocytes were acquired from HOD mice were opsonized in vitro with various concentrations of 4B7, 5B9, CBC512, MIMA29 and anti-OVA vs TER119 (as a positive control). Samples were then washed and incubated in species specific R-PE-conjugated secondary antibody (1:200) for 30 min at room temperature. Samples were then analyzed by flow cytometry to determine the mean fluorescence intensity (MFI) of the antibody-opsonized erythrocytes. (n= 3 per group)

### 6.3.3 Opsonized erythrocyte-macrophage binding assay
In order to determine whether the lack of phagocytosis seen with some antibodies was due to the opsonized RBCs not interacting with Fc receptors on macrophages, a binding assay was carried out. Experiments were carried out using the same methods as with the erythrocyte phagocytosis experiments except the experiments were carried out on ice to prevent internalization of RBCs.
and RBCs were not lysed at the end of the experiment. Unbound RBCs were removed by washing cells three times with PBS. It is notable that only TER119, 34-3C and M1/69 which could induce phagocytosis, were also able to bind to macrophages (Figure 18). Potentially suggesting that antibodies such as MIMA29 and CBC512 are unable to induce phagocytosis due to insufficient binding and/or activation of Fc receptors on macrophages despite their ability to bind efficiently to RBCs.
Figure 18. Ability of opsonized erythrocytes to bind to macrophages
Erythrocytes were acquired from HOD mice and either non-opsonized (control) or opsonized in vitro with the indicated antibodies then incubated with RAW264.7 macrophages on ice for 30 minutes. Binding efficiency was calculated using the equation in section 5.10 of the methods section. (n= 4 per group) **P<0.05, ***P<0.001

6.3.4 MIMA29’s ability to inhibit phagocytosis of TER119 opsonized RBCs

One last experiment was carried out to clarify why MIMA29 opsonized RBCs were not being phagocytosed. It is possible that the MIMA29 bound to the surface of RBCs was in fact interacting with Fc receptors, but possibly inhibitory receptors as opposed to activating ones thus giving us an unexpected result. We hypothesized that MIMA29 was interacting with Fc receptors and inhibiting the phagocytic process as opposed to not interacting at all. Therefore, using both MIMA29 and TER119 opsonized RBCs, we evaluated whether MIMA29 opsonized RBCs
would be able to inhibit the phagocytosis of TER119 opsonized RBCs, which are known to be phagocytosed. MIMA29 and TER119 opsonized RBCs were added either separately, together or MIMA29 RBCs were added first followed by TER119 RBCs. The results in Figure 19 demonstrate that when MIMA29 and TER119 opsonized RBCs are added at the same time there is not a decrease in phagocytosis of TER119 opsonized RBCs however, when MIMA29 opsonized RBCs are added 30 minutes before TER119 opsonized RBCs are there is a decrease in the phagocytic index. Therefore it is possible that MIMA29 acts as an inhibitor of phagocytosis.

![Active inhibition](image)

**Figure 19. Inhibition of phagocytosis of TER119 opsonized RBCs using MIMA29 opsonized RBCs.**

Erythrocytes were acquired from HOD mice and either non-opsonized (control) or opsonized in vitro with the indicated antibodies then incubated with RAW264.7 macrophages either together or separate for 30 minutes. Phagocytic index was calculated. (n= 3 per group) ***P<0.05

### 6.4 Assessment of antibody opsonized erythrocytes to prevent platelet phagocytosis in vitro

The primary theory for the mechanism of action of anti-D and other anti-RBC antibodies in ameliorating ITP is believed to be the MPS blockade theory. This occurs when opsonized RBCs compete with platelets for Fcγ receptors on macrophages thus causing an increase in platelet numbers$^{31,32}$. However, evidence supporting this theory is inadequate. In order to determine
whether the presence of opsonized RBCs can prevent phagocytosis of opsonized platelets, we have established an in vitro platelet phagocytosis assay using RAW 264.7 macrophages. Platelet phagocytosis was performed using select anti-erythrocyte antibodies (Table 7) in order to determine which antibodies would be able to reduce in vitro opsonized platelet phagocytosis. Phagocytosis assay were repeated 3x per antibody. A concentration of 5 µg/mL was selected for all of the antibodies because this was the concentration at which maximal phagocytosis was achieved, except 34-3C with which 40 µg/mL was used.

### Table 7. Anti-erythrocyte antibodies used to inhibit opsonized platelet phagocytosis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Reactivity</th>
<th>RBC binding</th>
<th>RBC clearance</th>
<th>Ameliorates ITP</th>
<th>Induces RBC phagocytosis</th>
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<td>TER119</td>
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</table>

1. RBC binding denotes how well the antibody bound to RBCs which was measured by flow cytometry (+++ = MFI > 500; +++ = MFI > 250; ++ = MFI > 125; + = MFI > 62.5; - = MFI < 62.5)

2. RBC clearance denotes how well an antibody was able to clear RBCs from circulation (++++ = > 50% clearance; +++ = > 25%; ++ = > 12.5%; + = > 6.25%; - = < 6.25%)

### 6.4.1 Platelet phagocytosis

In order to assess platelet phagocytosis, the fluorescent CellTracker Green CMFDA Dye was used to label platelets and platelets were opsonized using an anti-CD41 antibody (Mwreg30). To assure no background florescence would be detected with either of these products alone, we conducted experiments with each of these individually. The use of only either CMFDA or Mwreg30 was not enough to observe platelet phagocytosis or any florescence (Figure 20). When using both CMFDA and an anti-CD41 antibody (Mwreg30), platelets were observed to be internalized by RAW 264.7 cells (Figure 21). Antibodies towards platelet GPIIbIIIa are known
to induce phagocytosis of platelets via an Fc dependent manner. Antibodies towards GPIb are also seen in ITP patients, however they are said to function via an Fc-independent mechanism because IVIG is not effective in patients who have these antibodies. Therefore, an anti-GPIb antibody (Nit G) was also used in this experiment to determine whether Fc receptors are involved in the phagocytosis. As seen in Figure 21, Nit G is unable to induce complete phagocytosis in vitro seeing as most of the platelets remain on the periphery of the macrophages.

Figure 20. CMFDA or Mwreg30 alone are not sufficient to observe platelet phagocytosis. RAW 264.7 cells were cultured overnight, then platelets either opsonized with MWreg30 or non-opsonized and labeled with CMFDA were added to the media for 30 minutes at 37°C. **Left Panel** RAW 264.7 cells alone without any platelets. **Center Panel** RAW 264.7 cells incubated with CMFDA labeled non opsonized platelets. **Right Panel** RAW 264.7 cells incubated with Mwreg30 opsonized platelets not labeled with CMFDA. All samples were observed by confocal microscopy.
Figure 21. Phagocytosis of MWreg30 and Nit G opsonized platelets
RAW 264.7 cells were cultured overnight, then platelets labeled with CMFDA and either opsonized with MWreg30 or Nit G were added to the media for 30 minutes at 37°C. **Left Panel** RAW 264.7 cells with MWreg30 opsonized platelets. White arrow depicts an internalized platelet or platelet cluster. **Right Panel** RAW 264.7 cells incubated with Nit G opsonized platelets. White arrow depicts a surface bound platelet. All samples were observed by confocal microscopy.
6.4.2 TER119 opsonized RBCs prevent platelet phagocytosis in vitro

Once it was established that it was possible to observe platelet phagocytosis on the confocal microscope, it was possible to begin the experiments using antibody coated RBCs. TER119 was tested, as seen in Figure 22, it appears as though the presence of TER119 opsonized RBCs significantly reduces the amount of ingested platelets. Calculation of the phagocytic index confirmed that TER119 opsonized RBCs were able to reduce platelet phagocytosis by approximately 75% (Figure 23).
Figure 22. TER119 opsonized RBCs inhibit platelet phagocytosis in vitro
RAW 264.7 cells were cultured overnight, then platelets labeled with CMFDA and opsonized with MWreg30 were added with TER119 opsonized RBCs to the media for 30 minutes at 37°C. Top Left Panel RAW 264.7 cells alone. Top Right Panel RAW 264.7 cells incubated with MWreg30 & CMFDA platelets. Bottom Left Panel RAW 264.7 cells with MWreg30 & CMFDA platelets with non-opsonized RBCs. Bottom Right Panel RAW 264.7 cells incubated with MWreg30 & CMFDA platelets with TER119-opsonized RBCs. All samples were observed by confocal microscopy.
Figure 23. Phagocytic index of platelets incubated with TER119 opsonized erythrocytes

RAW 264.7 cells were cultured overnight, then platelets labeled with CMFDA and opsonized with Mwreg30 were either added to RAW cells with or without TER119 opsonized RBCs for 30 minutes at 37°C. Platelet phagocytic index was calculated using equation in section 5.12.7 of the methods section. ***(P<0.05. (n=5 per group)***
6.4.3 Some anti-RBC antibodies can prevent platelet phagocytosis in vitro

Following TER119 results, the anti-Duffy antibodies, MIMA 29 and CBC512, were examined for their ability to prevent platelet phagocytosis. These two antibodies cause RBC clearance but are unable to induce RBC phagocytosis in vitro. RBCs were opsonized and added to RAW macrophages along with platelets. Both of these antibodies were unable to significantly inhibit platelet phagocytosis (Figure 24).

The next antibody examined was the anti-Band-3 antibody 34-3C. This antibody both ameliorates ITP in a passive mouse model and causes anemia. It was not able to prevent phagocytosis at 5µg/mL but was observed to prevent platelet phagocytosis at a concentration of 40 µg/mL by approximately 55% (Figure 24).

Finally, M1/69 opsonized RBCs were analyzed for their ability to prevent platelet phagocytosis in vitro. This antibody both ameliorates ITP in a passive mouse model and causes RBC clearance. It was observed to prevent platelet phagocytosis at a concentration of 40 µg/mL by approximately 85% (Figure 24).
Figure 24. Anti-erythrocyte antibody coated RBCs ability to inhibit platelet phagocytosis. Erythrocytes were either non-opsonized or opsonized with antibody TER119, deglycosylated TER119, 34-3C (5 or 40 µg), M1/69, MIMA29 and CBC512 for 1 hour then incubated with RAW 264.7 cells and MWR-Reg30 opsonized CFMDA labelled platelets for 30 minutes. Cells were visualized by confocal microscopy and internalized platelets were counted by Imaris 8.0.2. Only TER119, 34-3C and M1/69 were able to significantly inhibit platelet phagocytosis in vitro. (P<0.05). (n=4-6 per group)
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Reactivity</th>
<th>RBC binding</th>
<th>RBC clearance</th>
<th>Ameliorates ITP</th>
<th>Causes RBC Phagocytosis</th>
<th>Inhibits platelet Phagocytosis</th>
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<td>TER119</td>
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<td>Rat IgG2&lt;sub&gt;b&lt;/sub&gt;</td>
<td>CD24</td>
<td>+++</td>
<td>+++</td>
<td>Yes/No</td>
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<td>YES</td>
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<tr>
<td>4B7</td>
<td>Mouse IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>HEL</td>
<td>+</td>
<td>-</td>
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<td>NO</td>
<td>N/T</td>
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<tr>
<td>5B9</td>
<td>Mouse IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>HEL</td>
<td>+</td>
<td>-</td>
<td>No</td>
<td>NO</td>
<td>N/T</td>
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<tr>
<td>MIMA29</td>
<td>Mouse IgG&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Fy3</td>
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<td>+++</td>
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<tr>
<td>CBC512</td>
<td>Mouse IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Fy3</td>
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<td>NO</td>
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<tr>
<td>Anti-OVA</td>
<td>Rabbit polyclonal</td>
<td>OVA</td>
<td>++</td>
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<td>No</td>
<td>NO</td>
<td>N/T</td>
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<tr>
<td>6A7</td>
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<td>hGPA</td>
<td>++</td>
<td>-</td>
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<td>NO</td>
<td>N/T</td>
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</table>

1. RBC binding denotes how well the antibody bound to RBCs which was measured by flow cytometry (++++ = MFI > 500; +++ = MFI > 250; ++ = MFI > 125; + = MFI > 62.5; - = MFI < 62.5)

2. RBC clearance denotes how well an antibody was able to clear RBCs from circulation (++++ = > 50% clearance; +++ = > 25%; ++ = > 12.5%; + = > 6.25%; - = < 6.25%)
Chapter 6

7 DISCUSSION

7.1 The relationship between in vivo RBC clearance and in vitro phagocytosis of opsonized RBCs

Anti-D, as well as some of the antibodies used in these experiments are able to cause anemia, which is an undesirable side effect of treatment. The mechanism by which this occurs is unclear and better knowledge as to the mechanism could hold some insight as to how clearance could potentially be removed from a future therapy. To expand our insight on the mechanism of the therapeutic activity of anti-erythrocyte antibodies in murine passive ITP, we employed the use of in vitro RBC and platelet phagocytic assays using RAW 264.7 cells.

It is believed that RBC clearance mediated by anti-erythrocyte antibodies could be occurring via Fcγ receptor mediated phagocytosis\(^\text{32}\) and as a result, antibodies which cause in vivo RBC clearance should allow for in vitro phagocytosis of opsonized RBCs. For the erythrocyte phagocytosis assays, out of 9 erythrocyte specific antibodies (Table 6) examined, 6 may have been unable to sufficiently opsonize RBCs for phagocytosis while the other 3 were found to better opsonize RBCs for phagocytosis. The antibodies which were poor opsonizers of RBCs for phagocytosis include: 4B7, 5B9, anti-OVA, 6A7, MIMA29 and CBC512. The anti-HEL antibodies (4B7 and 5B9), anti-OVA and the anti-human glycophorin A antibody (6A7) do not cause RBC clearance in mice and bind to a lesser degree to RBCs compared to the other antibodies (Figure 17), therefore it was not expected for them to sufficiently opsonize RBCs for phagocytosis. However, CBC512 and MIMA29 cause RBC clearance in mice and it is inconsistent with our first hypothesis to observe a lack of phagocytosis of erythrocytes opsonized with these antibodies. Further experiments were carried out using these antibodies to determine possible explanations for the lack of phagocytosis observed. These will be discussed in the next section.

The three antibodies which were able to better opsonize RBCs for phagocytosis by macrophages include: TER119, 34-3C and M1/69 (Figure 10, Figure 14 and Figure 15). These antibodies cause significant anemia in C57BL/6 mice and are also able to ameliorate ITP in a passive mouse model\(^\text{71,90}\). This result is consistent with our hypothesis that antibodies which cause in vivo RBC
clearance will sufficiently opsonize RBCs for phagocytosis by macrophages. TER119-opsonized RBCs had the highest phagocytic index of all the antibodies and therefore we used this antibody to determine whether Fc receptors were involved in the phagocytosis of RBCs opsonized with anti-RBC antibodies. To examine the functional importance of the Fc domain in the phagocytosis of TER119 opsonized RBCs, deglycosylated TER119 was generated (Dr. Ben Yu) using PNGase F. The Fc domain of antibodies is known to mediate multiple IgG effector functions and many of these rely on the conservation of the Fc N-glycan. Erythrocytes opsonized with this deglycosylated form of TER119 were unable to be phagocytosed which demonstrates that fully functional Fc regions seem to be required for phagocytosis of TER119 opsonized RBCs (Figure 11). This result is controversial in light of previous data which found that TER119 induced anemia occurs in C57BL/6 mice genetically engineered to lack the activating FcγRs. Together, these data suggest however the possibility that TER119 could induce RBC clearance via multiple pathways involving both its antigen-binding domain and its Fc domain.

In summary, it is unclear what the relationship is between an antibody’s ability to opsonize RBCs for phagocytosis and in vivo RBC clearance. Although, some anti-RBC opsonized RBCs (TER119, 34-3C and M1/69) were able to be phagocytosed, others were not. In these experiments, only nine antibodies were used and it may be that more antibodies need to be tested to be able to examine a trend. As mentioned, it was unexpected that anti-Duffy opsonized HOD RBCs were not phagocytosed seeing as these antibodies were also able to cause anemia in mice. A closer examination of both anti-Duffy antibodies was conducted and this is discussed in the following section.

**7.2 Anti-Duffy antibodies’ did not promote opsonized RBC phagocytosis despite their ability to induce anemia**

Despite the fact that CBC512 and MIMA29 are able to cause anemia in mice, they are unable to trigger RBC phagocytosis. This is contradictory with our first hypothesis which suggested that all antibodies that cause RBC clearance in vivo would also opsonize RBCs for phagocytosis. Further experiments would be helpful to substantiate this finding.

In these experiments, the standard ratio of macrophages to erythrocytes used was 20 erythrocytes per macrophage (1:20). This ratio has been reported in several publications examining phagocytosis however ratios as high as 1:100 have also been reported. To determine
whether the lack of phagocytosis seen with MIMA29 and CBC512 opsonized RBCs was due to an insufficient ratio of erythrocytes to macrophages, ratios of 1:20, 1:40 and 1:80 were used (Figure 16). An increase in the ratio of RBCs to macrophages used in the phagocytosis assay did not result in the phagocytosis of anti-duffy antibody opsonized RBCs but conversely resulted in a decrease of the phagocytosis of TER119 opsonized RBCs which confirm that ≤1:20 is the appropriate ratio (Figure 16).

Phagocytosis of opsonized RBCs would logically be dependent on the anti-erythrocyte antibody binding to the antigen expressed on the erythrocytes; therefore we examined the extent of binding of the antibodies to HOD RBCs. Both MIMA29 and CBC512 at 5 µg/ml had a higher degree of binding (measured by MFI) than 34-3C, an antibody of the same isotype as MIMA29, which is able to opsonize RBCs for phagocytosis (Figure 17). MIMA29 also had a similar MFI as compared to TER119, although these two antibodies were from different species (mouse and rat respectively) and we therefore used different secondary antibodies, therefore the results aren’t necessarily comparable. It appears as though MIMA29 and CBC512 bind sufficiently to HOD RBCs but this does not explain why RBCs opsonized with these antibodies are not phagocytosed.

The next hypothesis was that the antibodies, once bound to the surface of RBCs, were not interacting with Fc receptors on the macrophages. Incubation in the cold (≤ 4°C) was used to inhibit phagocytosis, therefore making it possible to observe surface bound RBCs prior to their phagocytosis. MIMA29 and CBC512 opsonized RBCs were not observed to bind to macrophages, however TER119, 34-3C and M1/69 which could sufficiently opsonize RBCs for phagocytosis, were able to bind to macrophages (Figure 18). These findings suggest that MIMA29 and CBC512 opsonized RBCs are potentially unable to be phagocytosed due to insufficient binding to Fc receptors on macrophages. The Fy3 region of the HOD antigen (to which MIMA29 and CBC512 bind) has been localized (Figure 2). Seeing as the antigen binding site is in close proximity to the plasma membrane of the cell it might make physical attachment of the bound antibody to the Fc receptor difficult and thus prevent phagocytosis. Antibodies to the other portions of the Duffy molecule would be helpful in determining whether binding site location is important.
There exist two types of Fc receptors, activating and inhibitory. Activating Fc receptors contain, or associate with molecules containing immunoreceptor tyrosine-based activation motifs (ITAM), when the Fc receptor comes in contact with an antibody, the relevant ITAMs are phosphorylated. This ITAM phosphorylation initiates downstream signaling pathways which result in cell activation and initiation of effector responses including phagocytosis. However, inhibitory receptors contain immunoreceptor tyrosine based inhibition motifs (ITIM) which when phosphorylated inhibits cell activation. We hypothesized that perhaps MIMA29 was interacting with an inhibitory Fc receptor, such as FcγRIIB, and preventing phagocytosis by active inhibition. To test this we used a phagocytosis assay to determine whether MIMA29 opsonized RBCs could prevent the phagocytosis of TER119 opsonized RBCs. Based on these experiments, MIMA29 opsonized RBCs decreased the phagocytic index of TER119 opsonized RBCs when they were incubated with RAW 264.7 cells 30 minutes before adding TER119 opsonized RBCs (Figure 19). However, this result could be attributed simply to the addition of opsonized RBCs in general therefore, we cannot fully attribute this effect to MIMA29. When the two subsets, TER119 and MIMA29 opsonized RBCs, were added at the same time the inhibitory effect was not observed. Therefore, there is a slight possibility that MIMA29 interacts with inhibitory Fc receptors to prevent phagocytosis of RBCs as opposed to not interacting at all, however more experiments need to be conducted in order to draw a conclusion.

In addition to these possibilities, there are many other possible explanations that exist as to why MIMA29 and CBC512 opsonized RBCs are not phagocytosed. For one, many of the antibodies used are of different isotypes and certain isotypes could be better than others at initiating a phagocytic response. However, the anti-band-3 antibody (34-3C) is of the same IgG2a isotype as MIMA-29 and 34-3C was able to opsonize RBCs for phagocytosis. I concluded therefore that it is unlikely that the isotype of the antibody was the reason for the lack of phagocytosis seen with MIMA29.

Second, perhaps MIMA29 induces anemia in an Fc receptor-independent manner. The two main pathways in clearance of antibody-coated RBCs in vivo are considered to involve either FcγRs or complement. Alternative pathways have also been described such as agglutination in vivo, membrane distortions, opening of calcium channels, and exposure of phosphatidylethanolamine due binding of some polyclonal human IgG antibodies. However, it was previously demonstrated by the Zimring lab that clearance of HOD RBCs by the anti-Fy3 antibody,
MIMA29, was not observed in mice genetically engineered to lack activating FcγR function, suggesting that FcγRs play a role\(^9^3\). Therefore it might seem unlikely that MIMA29 acts in an Fc independent manner to clear RBCs.

Another factor to consider is that the HOD antigens are transgenically expressed on the surface of HOD RBCs which could make them less stable than the endogenous antigens such as mouse GPA and Band-3. Antigen shedding is a phenomenon which occurs with the HEL antigen on the HOD RBC\(^{10^3}\). It is possible that antigens are being cleaved before they are recognized by macrophages. In addition there might not be enough antigens present on the cell surface to initiate a phagocytic response. This could explain the lack of phagocytosis of RBCs coated with HOD or hGPA specific antibodies as well as the low binding to HOD RBCs seen by 4B7 and 5B9.

Finally, it has also been found that in the case of platelet phagocytosis, C reactive protein in serum is required for phagocytosis\(^{10^4}\). Perhaps it’s the same case for erythrocyte phagocytosis yet some antibodies are able to cause phagocytosis regardless of the presence of serum factors.

In summary, it is possible that the lack of phagocytosis of anti-Duffy opsonized RBCs could be due to a lack of binding to FcRs on macrophages. With MIMA29 in particular we have observed that RBCs opsonized with this antibody are able to inhibit the phagocytosis of TER119 opsonized RBCs which suggest this antibody could be interacting with inhibitory Fc receptors. It is also possible that MIMA29 and CBC512 cause RBC clearance by some mechanism that hasn’t yet been explored. We have answered some questions but also given rise to many others which need to be addressed. However, in order to continue along with the original hypotheses we continued with the platelet phagocytosis experiments.

7.3 Anti-erythrocyte antibodies’ therapeutic ability is related to inhibition of platelet phagocytosis

The long held principle mechanism by which anti-D works in ITP is considered to be MPS blockade, a theory based on competitive inhibition whereby antibody-sensitized erythrocytes outnumber antibody-sensitized platelets and the erythrocytes are destroyed by splenic macrophages resulting in a decrease in the splenic sequestration/destruction of auto-antibody.
sensitized platelets (Figure 1). Even though MPS blockade constitutes the originally proposed mechanism as to how anti-D works, there is very little direct data to support this hypothesis.\(^{32}\) The MPS blockade model of anti-D proposes that erythrocytes are destroyed by macrophages through an Fcγ receptor-dependent mechanism thus limiting the number of Fc receptors available for opsonized platelets. If MPS blockade is in fact the primary mechanism through which anti-erythrocyte antibodies work in ITP, a decrease in platelet phagocytosis should be observed in vitro when both opsonized platelets and erythrocytes are added simultaneously to macrophages.

Having identified which anti-erythrocyte antibodies were able to successfully opsonize RBCs for phagocytosis (Table 6), we performed platelet phagocytosis experiments using selected antibodies (Table 7). Anti-erythrocyte antibody opsonized RBCs were then tested for their ability to inhibit platelet phagocytosis in the platelet phagocytosis assay. TER119, 34-3C and M1/69 opsonized RBCs were all able to inhibit platelet phagocytosis by a significant margin which may have to do with the fact that these are able to be phagocytosed by macrophages (Figure 24). No other antibody opsonized RBCs were able to inhibit platelet phagocytosis. This is a promising result seeing as these three antibodies (TER119, 34-3C and M1/69) are also able to ameliorate ITP in a passive mouse model. This result also coincides with our hypothesis that antibodies which ameliorate ITP will inhibit platelet phagocytosis. This could indicate that the mechanism of anti-erythrocyte antibodies in the amelioration of ITP is related to their ability to successfully interact with Fc receptors on macrophages thus initiating phagocytosis of opsonized RBCs and inhibiting platelet phagocytosis. If we can predict which antibodies will function in ITP by using a simple phagocytosis assay this could potentially save researchers and patients a significant amount of time and money when it comes to identifying novel therapies for ITP.
8 Summary

In conclusion, we note the fact that not all anti-erythrocyte antibodies which are able to induce RBC clearance can opsonize RBCs for phagocytosis which is in disagreement with our first hypothesis. When the anti-Duffy antibodies MIMA29 and CBC512 were used to opsonize RBCs phagocytosis was not observed which could potentially due to inadequate binding of the antibody to Fc receptors on macrophages or the fact that MIMA29 interacts with inhibitory Fc receptors to prevent a cellular response. Further study is warranted to determine the exact mechanism for how these antibodies induce RBC clearance. In addition, using deglycosylated TER119 we were able to determine that phagocytosis of TER119 opsonized RBCs requires the Fc region.

Our second hypothesis appears correct, as all antibodies that ameliorate ITP in a passive mouse model also inhibit platelet phagocytosis in the in vitro model. Our data represents the first study of a platelet phagocytosis model using anti-erythrocyte antibodies to support the MPS blockade theory. This data is significant because it suggests that there is a potential methods with which we may be able to predict which anti-erythrocyte antibodies will function in ITP. This could be very cost effective in the long run if only antibodies which function in this assay move onto clinical trials, which is better for both patients and researchers.
Chapter 10

9 Future Directions

Our data conclusively shows that anti-erythrocyte antibodies have the ability to opsonize RBCs for phagocytosis and the ability to prevent platelet phagocytosis. However, exactly which anti-erythrocyte antibodies have these properties, and how they are being achieved, is of considerable interest. As such, we propose the following future experiments to help address these questions:

1) Conduct phagocytic inhibition experiments using MIMA29 and CBC512 and the proper controls to determine if they interact with inhibitory Fc receptors.

2) Conduct experiments using C-reactive protein to determine if the addition of the serum factor will allow for phagocytosis of all opsonized RBCs.

3) Analyze other erythrocyte specific antibodies for their ability to ameliorate ITP and prevent platelet phagocytosis to determine if they follow the same trend discovered in this thesis.
REFERENCES


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Supplemental Figure 1. Anti-OVA IgG dose response study in HOD mice.

**Left panel.** Mice were uninjected (■—■) or injected with 30 µg of antibody TER-119 (●—●) or anti-OVA antibody at 30 µg (○—○), 60 µg (□—□), and 120 µg (Δ—Δ). Mice were bled and erythrocytes enumerated at the time points shown. (n=1 per dose) **Right panel.** Erythrocytes from the same mice were evaluated for the relative level of in vivo anti-OVA antibody binding by reacting the erythrocytes with a goat anti-rabbit R-PE conjugated secondary antibody. The MFI shown corresponds to the doses used on the left. Performed in collaboration with Mark Jen.
**Supplemental Figure 2.** Anti-HEL antibody dose-response studies in HOD mice.

Mice were injected with 30 µg of antibody TER-119 (●---●) or anti-HEL antibodies (Δ--Δ): 8E12, 6D7, 5B9, 4B7, and 6F7 at 3, 30, 60, and 120 µg/mouse as indicated. Mice were also injected with mouse IgG (▲—▲) at 3, 30, 60, and 120 µg/mouse which correspond to the dose of anti-HEL antibody used in each figure. Mice were bled and erythrocytes were enumerated at the time points shown. This was done in collaboration with Mark Jen. (n=1 per dose).