Murine Model of Autoimmune Hemolytic Anemia: Red Blood Cell Clearance Mechanisms and Treatment Efficacy

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
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University of Toronto
2012

Abstract

Antibodies against erythrocyte-specific antigens can cause immune red blood cell (RBC) destruction, resulting in autoimmune hemolytic anemia (AHA). Therapy for patients with AHA remains a challenge, and better knowledge of pathophysiological mechanisms is needed. In this thesis, the mechanisms of RBC clearance mediated by a panel of monoclonal anti-mouse RBC antibodies were characterized in a murine model of passive AHA, and efficacy of treatments was evaluated. It is demonstrated herein that mechanisms in addition to the classical macrophage Fcγ receptor (FcγR)- and/or complement-mediated hemolysis may be contributing to the development and/or persistence of anemia. The efficacy of intravenous immunoglobulin (IVIg) therapy varies in relation to the mechanism of anemia in mice with FcγR-mediated anemia being highly related to IVIg responsiveness. In addition, this thesis shows that monoclonal RBC-specific antibodies can ameliorate thrombocytopenia in a murine model of passive immune thrombocytopenia (ITP) analogous to the use of anti-D in ITP patients.
I would first like to thank my supervisor, Dr. Alan Lazarus, for having always believed in and encouraged me. I feel so lucky to have shared his incredible knowledge, wisdom and optimism both in science and in life, and will be forever indebted to him for his support professionally, financially and personally over the years.

A heart-felt thanks to all the past and present members of the Lazarus lab: Andrew, Sara, Nancy, Zhongwei, Joan, Pat, Honghui, Lidice and Alaa for your help and support to me, and most importantly, for all the laughter we shared in and out of the lab.

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<th>Description</th>
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<tbody>
<tr>
<td>AHA</td>
<td>autoimmune hemolytic anemia</td>
</tr>
<tr>
<td>CA</td>
<td>cold agglutinin</td>
</tr>
<tr>
<td>CAS</td>
<td>cold agglutinin syndrome</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CPDA</td>
<td>citrate-phosphate-dextrose solution with adenine</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc γ receptor</td>
</tr>
<tr>
<td>GPA</td>
<td>glycophorin A</td>
</tr>
<tr>
<td>GPC</td>
<td>glycophorin C</td>
</tr>
<tr>
<td>HDN</td>
<td>hemolytic diseases of the newborn</td>
</tr>
<tr>
<td>HSA</td>
<td>heat stable antigen</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IHA</td>
<td>immune hemolytic anemia</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>ITP</td>
<td>immune thrombocytopenia</td>
</tr>
<tr>
<td>IVIg</td>
<td>intravenous immunoglobulin</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MBL</td>
<td>mannose-binding lectin</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>NZB</td>
<td>New Zealand Black</td>
</tr>
<tr>
<td>PCH</td>
<td>paroxysmal cold hemoglobinuria</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet-rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>TRALI</td>
<td>transfusion-related acute lung injury</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1 Introduction

Autoimmune hemolytic anemia (AHA) is an acquired immunologic disease characterized by destruction of red blood cells (RBC) as a result of production of antibodies directed against self RBC antigens. AHA is arguably the oldest recognized autoimmune condition in humans [1, 2], yet a relatively uncommon disease with annual incidence estimated at 1~3 per 100,000 [3]. The majority of cases of AHA are mediated by warm-reactive autoantibodies, defined by antibodies that exhibit maximal RBC reactivity at 37˚C, and therefore referred to as warm AHA (wAHA). In contrast, cold agglutinin syndrome (CAS) is caused by antibodies that display optimal RBC reactivity at 4˚C. Patients with warm and cold antibodies detected in their sera that satisfy the diagnostic criteria for both wAHA and CAS are said to have mixed AHA. Another AHA subtype is paroxysmal cold hemoglobinuria (PCH). PCH is distinct from CAS in that the causative antibody requires exposure to cold temperature followed by exposure to 37˚C environment in order for the hemolysis to occur. Finally, cases have been reported where ingestion of a drug causes the development of RBC autoantibodies; such cases are termed drug-induced AHA. Alternatively, AHA can be classified as being primary (idiopathic) if it is not associated with an underlying disorder and secondary if an association with an additional disease is established.

The distinction between different types of AHA is important for prognosis and management purposes. The most common type of AHA, wAHA represents 70~90% of all cases [4, 5], and is associated with the poorest prognosis with significant mortality [6]. Typically of immunoglobulin G (IgG) isotype, warm autoantibodies used to be considered nonspecific and bind to all the common types of human RBC in blood bank test panels. Recent reports, however, suggest that binding is specific but to “public” antigens common to almost all human RBC and thereby appear to be panreactive [6]. It has been demonstrated that autoantibody specificities in
about half of the patients are predominantly directed against Rh antigens such as e, E or C, and other specificities identified include epitopes on glycophorins, membrane band 3 protein, the Kell, Kid, Duffy, and ABO systems [7, 8].

In addition to antibody specificities, other characteristics of the bound antibody including quantity, ability to fix complement and ability to bind to tissue macrophages are all determinants of the degree and mechanism of hemolysis. The current understanding of mechanisms of immune RBC destruction is that two distinct types of hemolysis exist. The term intravascular hemolysis describes complement-mediated RBC lysis directly in the blood stream resulting in hemoglobinemia and likely hemoglobinuria, whereas the term extravascular hemolysis is used when RBC is instead destroyed within the reticuloendothelial system (RES) as a result of the recognition of the RBC-bound antibodies or complement components by their respective receptors expressed by macrophages of the RES. Despite the fact that intra- and extra-vascular hemolysis are considered the principle immune RBC destruction mechanisms, there are emerging clinical findings that seem to challenge the absolute requirement for complement or macrophage classical phagocytosis pathways [9, 10]. Indeed, several novel mechanisms have been proposed to explain individual cases yet are hard to reproduce due to the rare but potentially very heterogeneous nature of the disease. These mechanisms are discussed in subsequent chapters of this thesis.

Parallel to an incomplete understanding of the RBC destruction mechanisms, management of AHA remains regrettably experience-based in the era of evidence-based medicine [11]. Newly diagnosed primary wAHA is managed with first-line treatment of glucocorticoids (steroids), to which approximately 70-80% of patients will respond, but only 15-20% will achieve complete remission and remain steroid free. Patients who become refractory to steroid therapy are
considered for second-line treatment that includes the options of splenectomy, cytotoxic drugs, and in recent years, the anti-CD20 antibody rituximab. Most if not all of these therapies have low curative potential, but severe and usually long term side effects [7, 11].

The efficacy of intravenous immunoglobulin (IVIg) treatment of immune thrombocytopenia (ITP), which is an autoimmune condition characterized by platelet destruction mediated by platelet specific autoantibodies, was established in the 1980s [12, 13] and suggested that the IVIg treatment of AHA might offer an alternative to the conventional treatments but with considerable lower risk of side effects. However, reported efficacy has been inconsistent [14-17], comparing unfavorably with IVIg treatment of ITP and of autoimmune neutropenia, and with other treatment modalities for AHA [18]; as a result, IVIg remains off-label for this indication [19]. In order to identify patient variables useful for identifying responders to IVIg, a controlled clinical trial will be required; yet such studies are generally lacking with AHA patients due to the low incidence of the disease and numerous confounding factors such as additional underlying disorder (in the case of secondary AHA) and the fact that most patients have received or are receiving multiple concurrent treatments.

In summary, AHA is an autoimmune disorder with potentially severe consequences. The pathological mechanisms are not fully understood, and the current treatment for AHA patients is not satisfactory. In this thesis, we developed a mouse model of passively induced AHA with a panel of monoclonal mouse RBC-specific antibodies, and used this model as a platform to study the mechanism of RBC destruction and efficacy of treatment. These results where we examined the pathologic effects of these RBC-specific antibodies are presented in Chapters 4 and 5. In Chapter 6 we explored the therapeutic potential of RBC clearance, and studied these monoclonal
RBC antibodies for their ability to ameliorate thrombocytopenia in a murine model of passively induced immune thrombocytopenia (ITP).
Chapter 2

Literature Review
2 Literature review

2.1 Mechanisms of RBC clearance

2.1.1 Overview

One’s total RBC volume can only be decreased rapidly by two mechanisms: bleeding (blood loss) or RBC hemolysis. A cessation of RBC production in a patient with a normal RBC life span, on the other hand, can only result in a decrease in RBC count of a hardly detectable 1% per day, since the normal RBC life span is ~120 days in the circulation in human [20]. The precise mechanism of recognition of senescent and injured RBC is not fully understood. One theory suggests that erythrocyte aging may lead to binding of autologous IgG recognizing a senescent antigen residing on the band 3 membrane protein and subsequent removal of the IgG-bound RBC through phagocytosis mediated by the macrophages of the RES [21]. Alternatively, some propose a mechanism of caspase-independent RBC apoptosis, or “eryptosis”, where the aged red cells experience a series of changes including increased Ca$^{2+}$ permeability, cell shrinkage, reduced CD47 expression, and exposure of the inner membrane phospholipids that are recognized by macrophages equipped with the corresponding receptors [22, 23].

RBC clearance as a result of autoantibody-coated RBC in AHA is mediated by different mechanisms than in senescent RBC clearance. The current understanding of mechanisms of immune RBC destruction is that two distinct, but not exclusive, types of hemolysis exist [6]. The dominant type of extravascular hemolysis describes the macrophage-mediated RBC destruction that predominantly takes place in macrophages of the RES in the spleen and liver. Intravascular hemolysis, on the other hand, describes the complement-mediated direct lysis of antibody-coated
RBC in the blood stream. These two mechanisms are reviewed in detail in the following sub-sessions. Despite the fact that intra- and extra-vascular hemolysis are considered the principle immune RBC destruction mechanisms, there are emerging clinical findings that seem to challenge the absolute requirement for and involvement of complement or macrophage classical phagocytosis pathways [24]. Indeed, several novel mechanisms have been proposed and will also be discussed.

2.1.2 Extravascular hemolysis

The principle mechanism of hemolysis in IHA is considered to be the opsonization of the red cells by erythrocyte-specific antibodies as well as complement components, followed by recognition by the Fc- and complement receptors, respectively, expressed by the splenic macrophages and liver Kupffer cells, leading to phagocytosis of these cells and consequently, extravascular hemolysis [25]. Both IgG and IgM antibodies can be involved, although likely via different mechanisms. IgM in its pentameric form engages the classical complement pathway most efficiently among all immunoglobulins. As the early complement components C3b and iC3b are activated, they act to opsonize the IgM-sensitized red cells and mediate the subsequent phagocytosis [26]. On the other hand, the IgG antibodies, considered relatively poor activators of the complement pathway, are readily recognized by the Fc receptors on the phagocyte surface to initiate erythrophagocytosis. Interestingly, there is evidence that the IgG antibody and complement fragments may act in a synergistic manner to enhance phagocytosis when both are present on the RBC [27-29].
2.1.3 **Intravascular hemolysis**

Intravascular hemolysis in AHA is caused by complement-mediated immune hemolysis. Among the three pathways of complement activation: classical, the alternative and the mannose-binding lectin (MBL) pathways [30], the classical pathway appears to be the most relevant pathway in causing intravascular hemolysis in immune mediated RBC destruction, whereas evidence indicating a role of the other two pathways is rare [6]. Activation of late components of the complement cascade is required, most likely by IgM antibodies, the membrane attack complex (MAC, composed of complement C5-C9) is formed, and the target RBC are lysed within the circulation, therefore causing intravascular hemolysis. The lysed RBC then release hemoglobins into the plasma which are filtered through the glomerulus, and the patient is presented with hemoglobinemia and hemoglobinuria.

2.1.4 **Multivalency-mediated hemagglutination**

Work done in a passive mouse model of AHA has revealed yet another possible mechanism of multivalency-mediated hemagglutination. Using monoclonal anti-RBC autoantibodies derived from the New Zealand Black (NZB) mouse, Shibata et al [31] showed that the IgM autoantibodies derived from these mice did not cause significant phagocytosis, nor did they stimulate complement-mediated lysis. Instead, these IgM monoclonal antibodies markedly agglutinated mouse RBC both *in vitro* and *in vivo*, and caused severe anemia. Baudino et al [32] extended these findings by reporting that polymeric forms of IgM and IgA class-switch variants of the same antibody caused anemia as a result of hemagglutination and subsequent sequestration of the target RBC in the spleen, whereas their monomeric counterparts were free of any pathogenic effects. They also confirmed the lack of complement contribution in this process by
showing the similar degree of anemia developed in the C3-deficient mice. Furthermore, an IgA-mediated human AHA case was reported recently [10], where it was shown that hemagglutination in the spleen induced by the polymeric IgA antibody was likely responsible for the anemia, which was found to be independent of complement activation and Fc-mediated erythrophagocytosis.

2.1.5 *Anti-glycophorin A antibody-mediated RBC hemolysis*

Recently Brain et al [9, 33, 34] presented a series of case reports of life-threatening AHA due to autoantibodies that had a specificity for determinants on the RBC membrane glycoprotein, glycophorin A (GPA). They went on to show that *in vitro* incubation of erythrocytes with the GPA-specific antibody from the patients lead to hemolysis not via complement but via an enhanced RBC membrane permeability to the Na\(^+\) and Ca\(^{2+}\) cations, accompanied by increased phosphatidylethanolamine (PE) exposure. GPA is one of the 2 most abundant integral proteins in the erythrocyte membrane (the other is band 3), present at an estimated 1 million copies per cell [35], and carries several clinically relevant blood group antigens including M, N, Pr, and En\(^a\). The exact function of GPA remains unclear, although it carries the majority of the negative charges of RBC due to its high sialic acid content, and thereby likely contributing to the electrostatic repulsion that prevents RBC aggregation [36]. Of interest, reported AHA cases due to GPA-specific antibodies are relatively rare but usually severe, and Brain et al.’s findings suggest that mechanisms other than the canonical macrophage FcR/complement-mediated hemolysis may be involved.
2.1.6 **Anti-Kell antibody-mediated inhibition/destruction of erythroid progenitor cells**

The Kell blood-group system is one of the major antigenic systems in human RBC, and anti-Kell antibodies account for 10% of all cases of immune mediated severe fetal anemia in hemolytic disease of the new-born (HDN), second only to HDN mediated by anti-D [37]. Interestingly, the mechanism of fetal anemia caused by anti-Kell antibodies seem to differ from that of classic HDN associated with anti-D. Affected Kell-alloimmunized fetuses usually present with lower numbers of circulating reticulocytes and normoblasts, compared with anti-D-affected fetuses, despite an elevated level of erythropoietin released, and the inverse relationship between hemoglobin level and reticulocyte counts found in anti-D-affected cases was missing in anti-Kell-affected fetuses. These observations led to the hypothesis that anti-Kell antibodies target RBC precursor cells instead of mature RBC in the circulation. Consistent with this hypothesis, it was found that anti-Kell-affected cases have low serum bilirubin levels perhaps as a result of hemolysis of erythroid precursor cells which are non- or in-completely hemaglobinized [38]. In addition, Vaughan et al [39] demonstrated that monoclonal IgG and IgM antibodies are capable of specifically inhibiting the growth of Kell-positive (but not Kell-negative) RBC precursor cells in a dose-dependent manner, whereas anti-D had no such effect. Their findings were furthered by Daniels et al [40] who showed that Kell antigen appears on erythroid progenitor cells early in erythropoiesis, prior to the expression of glycophorin A and band 3. The authors went on to show that Kell-positive progenitor cells sensitized with anti-Kell sera from pregnant women elicited a strong response from monocytes *in vitro*, and proposed that anti-Kell but not anti-D may cause fetal anemia by promoting immune destruction of the Kell-positive erythroid early progenitor cells by macrophages in the fetal liver.
2.2 Mouse models of AHA

2.2.1 Overview

The best way to understand human disease is to study human patients, yet this is particularly difficult with AHA patients due to the low number of cases and the highly heterogeneous nature of the disease. As a result, most published reports contain information from very small populations, and results are often inconsistent between studies. In studies aimed to evaluate efficacy of treatments, this is further complicated by the fact that most AHA patients have received or are receiving multiple therapies for their anemia, making it difficult to determine the effect of individual medications.

Mouse models are widely used to understand basic pathogenic mechanisms of antibody-mediated RBC clearance [41, 42], and are the choice of animal model in this report. Mice are not humans, and there are some differences that need to be considered when extrapolating mouse data to the human system. For example, the mouse complement has been shown to be relatively inefficient in lysing erythrocyte targets compared to humans and other species [43, 44], and the IgG subclasses and Fc receptors are different in mice and humans [45, 46]. The mouse IgG-FcγR system will be briefly reviewed next, followed by an introduction to the various mouse models of AHA.

2.2.2 Mouse IgG-FcγR system

In mice there are four IgG subclasses: IgG1, 2a, 2b, and 3, and four classes of Fcγ receptors including the activating Fc receptors FcγRI, FcγRIII, FcγRIV, and an inhibitory receptor FcγRIIB (Fig. 1). The high affinity FcγRI is specific for IgG2a, the low affinity FcγRIII for IgG1,
2a, and 2b, and FcγRIV binds to IgG2a and 2b with intermediate affinity [47]. Using Ig class switch variants of an anti-mouse RBC antibody, Baudino et al [47, 48] documented the role of each activating Fc receptor in effector mechanisms mediated by different IgG subclass in a mouse model of AHA. Summary of their findings is presented in Table 1. All three activating FcRs share a common signal-transducing γ-chain (FcRγ), which is absent in the inhibitory FcγIIB; the γ-chain harbors the immunoreceptor tyrosine-based activation motif (ITAM) and is required for cell activation [46]. Both activating and inhibitory Fc receptors are expressed by the innate immune effector cells including monocytes, macrophages and dendritic cells (DC), whereas natural killer cells (NK) only express FcγIII, and the B cells solely express the inhibitory FcγIIB [49, 50]. For the purpose of dissecting the role(s) of each of the Fc receptors in the Fc-dependent RBC clearance mediated by different IgG subclasses, knockout mice deficient in the FcRγ or FcγRIIB, in combination with blocking antibodies directed against one or several of the FcγRs have proved powerful tools [41, 48].
Figure 1. The family of mouse Fc receptors for IgG. Mouse activating FcγRs consist of a ligand-binding α-chain and a signal-transducing γ-chain dimer, and it is the γ-chain that carries the immunoreceptor tyrosine based activating motifs (ITAM). On the other hand, the inhibitory FcγRIIB contains a single α-chain which contains the immunoreceptor tyrosine based inhibitory motif (ITIM). Figure from Nimmerjahn and Ravetch [46].
Table 1. The pathogenic activities of the Ig class switch variants of the mouse 34-3C anti-RBC antibody (modified from Baudino et al [48])

<table>
<thead>
<tr>
<th>Ig</th>
<th>Anemic dose*</th>
<th>Effector mechanisms</th>
<th>Pathogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>500 µg</td>
<td>FcγRIII</td>
<td>Erythrophagocytosis</td>
</tr>
<tr>
<td>IgG2a</td>
<td>25 µg</td>
<td>FcγRIII&gt;FcγRIV, Complement</td>
<td>Erythrophagocytosis</td>
</tr>
<tr>
<td>IgG2b</td>
<td>25 µg</td>
<td>FcγRIII&gt;FcγRIV, Complement</td>
<td>Erythrophagocytosis</td>
</tr>
<tr>
<td>IgG3</td>
<td>100 µg</td>
<td>Complement</td>
<td>Erythrophagocytosis</td>
</tr>
</tbody>
</table>

*Quantity of the monoclonal antibody required for inducing <40% hematocrit values.
2.2.3  **Mouse AHA models**

AHA can be induced in mice after introduction of xenogeneic RBCs. One classical example of this is the model developed by Playfair and Marshall-Clarke [51] that involves repeated immunization with rat RBCs. In these mice anti-mouse RBC autoantibodies eventually appear as well as the rat-specific xenoantibodies.

On the other hand, AHA can be readily studied in spontaneous mouse models such as the New Zealand Black (NZB) and the non-obese diabetic (NOD) mice [52, 53]. For example, the NZB mouse strain is genetically predisposed to develop a spontaneous and severe form of AHA, usually by 6 months of age, caused by pathogenic anti-mouse RBC autoantibodies [52]. One caveat of the use of these spontaneous models is that these mice also develop other autoimmune diseases as they age that also involve autoantibody formation (e.g., systemic lupus erythematosus or SLE in the NZB mice [54], and autoimmune insulin-dependent diabetes in the NOD mice [53]). Therefore, an alternative passive model has been developed and preferred, in which transfusion of the anti-RBC antibodies, or implantation of anti-RBC hybridomas are used to induce AHA with greater control [41]. For example, a passive model of monoclonal anti-RBC autoantibodies, derived from the NZB mice, and their Ig class switch variants has been used extensively to study the effector mechanism of IgM- or IgG-coated RBC clearance [32, 47, 48, 55-59]. Unlike in humans where autoantibodies against the RBC anion channel protein, band 3, are only produced in a minority of patients, band 3 is the dominant autoantigen in the NZB mice [60]. The most widely used monoclonal antibody derived from these mice, 34-3C, an IgG2a, has been consistently shown to cause FcγR-mediated erythrophagocytosis [47, 48].
Chapter 3

Rationale, Hypothesis and Specific Aims
3 Rationale, hypothesis and specific aims

1. Rationale

Current paradigm dictates that RBC clearance in immune-mediated hemolytic anemia primarily involves extravascular hemolysis (macrophage FcR/complement-mediated phagocytosis), intravascular hemolysis (complement-induced direct lysis), or a combination of both. However, recent clinical reports suggest that previous unrecognized mechanisms might be contributing to severe hemolysis [10, 24], in particular, in cases where GPA-specific antibodies are implicated [9, 10, 24, 33, 34], yet most of the clinical investigations are defined by in vitro work.

Hypothesis

In this thesis, it is hypothesized that different anti-RBC antibodies may mediate different RBC clearance mechanism(s), and that mechanism(s) in addition to the classical extra- and intra-vascular hemolysis may be involved in GPA antibody-mediated anemia.

Aims

The thesis aims to first optimize a murine model of passive AHA, and with this model, to examine RBC destruction attributable to the classical extra- and intra-vascular hemolysis mechanisms caused by a panel of monoclonal mouse erythrocyte-specific antibodies (chapter 4 and 5). Particular interest was placed on a GPA antibody to take advantage of both in vitro and in vivo system in the mouse model to elucidate potential novel pathological mechanism(s) (chapter 4).

2. Rationale
IVIg is used to effectively treat autoimmune diseases targeting blood cells such as immune thrombocytopenia (ITP) and autoimmune neutropenia (AIN), yet its use in the treatment of AHA remains controversial due to inconsistent efficacy, with no established explanation as to why some patients respond while others do not. Controlled clinical trials are needed to determine patient variables associated with treatment success yet such trials are extremely difficult because of the low number of patients and the heterogeneous patient population due to high prevalence of secondary conditions and concurrent medications.

**Hypothesis**

In this thesis, it is hypothesized that mechanisms of anemia induced by AHA-causing antibodies may be an important factor in determining response to IVIg therapy.

**Aims**

The thesis aims to test IVIg efficacy in ameliorating a murine model of passive AHA caused by a panel of monoclonal mouse erythrocyte-specific antibodies, and to determine if specific RBC destruction mechanism(s) is associated with IVIg responsiveness (Chapter 5).

3. **Rationale**

Another interest of the monoclonal mouse erythrocyte-specific antibodies lies in their use in the treatment of ITP. Polyclonal anti-D has long been established as an effective alternative to IVIg in the treatment of ITP in D-positive patients [61]. If monoclonal erythrocyte-specific antibodies prove effective in the amelioration of ITP, they would provide a cheaper and potentially safer substitution for IVIg and anti-D. However, a small
pilot study [62] reported a lack of beneficial response in 6 out of 7 patients tested, making it unethical for further trials in patients. Nevertheless, interest in monoclonal anti-D (or anti-D-like antibodies) was rekindled by recent findings in mouse studies where two monoclonal anti-mouse RBC antibodies were shown capable of ameliorating immune-mediated thrombocytopenia in a murine model of ITP [63]. However, controversy arises in one of these two antibodies as its efficacy was confirmed in some of the later publications [64, 65] but questioned in others [66]. In addition, the therapeutic mechanism of these erythrocyte-specific antibodies in the amelioration of ITP awaits further elucidation.

**Hypothesis**

In this thesis, it is hypothesized that monoclonal mouse erythrocyte-specific antibodies are of potential value in the treatment of thrombocytopenia in a murine model of ITP, and that they may work by different therapeutic mechanisms.

**Aims**

The thesis aims to evaluate the efficacy of a panel of mouse erythrocyte-specific antibodies in the amelioration of thrombocytopenia in a murine model of passive ITP, and investigate if the ability to mediate FcγR-mediated RBC clearance is required for efficacy (Chapter 6).
Chapter 4

Glycophorin A Antibody-Mediated Anemia in a Murine Model:
Minimal Involvement of FcγR or Complement,
and Implications for Agglutination
4 Glycophorin A Antibody-Mediated Anemia in a Murine Model: Minimal Involvement of FcγR or Complement, and Implications for Agglutination

4.1 Abstract

Autoimmune hemolytic anemia (AHA) is characterized by premature destruction of red blood cell (RBC) mediated by erythrocyte-specific antibodies. Therapy for patients with AHA is challenging and better knowledge of the involved pathophysiological mechanisms is needed. The classical paradigm of antibody-mediated RBC clearance in AHA involves macrophage Fc receptor (FcR)/complement-mediated phagocytosis, complement-induced direct lysis, or a combination of both. However, this paradigm has been challenged by intriguing case reports where anti-human GPA antibody was shown to cause hemolysis via mechanism(s) potentially independent of complement or macrophage FcR. In this study, using a murine model of immune hemolytic anemia (IHA), we investigated the pathophysiological mechanisms of GPA antibody-mediated anemia with a mouse GPA-specific antibody (TER119). The anemic effect of the IgG GPA-specific antibody was studied in vivo, by injecting the antibody into normal vs mice genetically deficient for the Fc γ-chain (required for activating FcγR function), complement C3, mice naturally deficient in complement C5 and splenectomized mice. In vivo pathologies were assessed by histological examination of peripheral blood and the spleen and liver of the reticuloendothelial system (RES). In addition, direct RBC destruction was evaluated in vitro by incubation of mouse RBC with the GPA antibody. We report that the GPA antibody induced significant anemia and an acute episode of hemoglobinuria in mice. It caused a similar degree of anemia in Fcγ+/ mice, C3+/ mice and C5-deficient mice, in comparison to control mice. Spleen weight was found correlated with the degree of RBC destruction, and splenectomized mice were
moderately but significantly delayed for anti-GPA-mediated anemia. Incubation of mouse RBC in vitro with increasing amount of GPA antibody was associated with increased propensity to agglutinate at 37°C vs 4°C, and blood smears revealed in vivo agglutination in the peripheral blood in GPA antibody-treated mice. Taken together, our results showed that an IgG GPA-specific warm antibody caused severe agglutination and anemia which persisted in both FcγR- and complement-deficient mice, and that splenectomy had a transient but significant effect in delaying this anemia.

4.2 Introduction

Allo- and auto-antibodies to blood group antigens can cause immune RBC destruction, resulting in hemolytic transfusion reactions or autoimmune hemolytic disease, such as autoimmune hemolytic anemia (AHA). The classical paradigm of antibody-mediated RBC clearance in AHA involves macrophage FcR/complement-mediated phagocytosis, complement-induced direct lysis, or a combination of both. However, this paradigm was recently challenged by intriguing case reports where anti-human GPA antibody was shown to cause anemia via mechanism(s) potentially independent of complement or macrophage FcR [9, 33, 34]. GPA is one of the 2 most abundant integral proteins in the erythrocyte membrane (the other is band 3), present at an estimated 1 million copies per cell [35], and is the major contributor to the erythrocyte’s negative charge due to its high sialic acid content [36, 67]. The exact function of GPA is still not fully understood. Complete loss of GPA on human RBC is not associated with detectable alterations in shape, function or lifespan of the red cells [68], but reduction in sialic acid has been demonstrated to enhance RBC aggregation [69], supporting a potential role of GPA in minimizing cell-cell interaction [36]. In addition, lectin- and antibody-induced ligation of GPA has been shown to reduce rotation and lateral mobility of band 3 [70], cause destabilization of the
phospholipid bilayer [33], and make RBC more resistant to deformation [71] that may result in splenic entrapment [72]. Of interest, reported AHA cases caused by antibody directed against epitopes on or associated with GPA (e.g., anti-M,-N,-Pr,-En) are relatively rare but usually severe [24].

In this study, we modeled anti-GPA antibody-mediated anemia with a monoclonal GPA-specific antibody, TER119, in a murine model of immune hemolytic anemia (IHA). This antibody is of interest because it can ameliorate passive immune thrombocytopenia (ITP) in mice analogous to the use of anti-D in ITP patients [63-65]. TER119 is widely used as a specific marker for the erythroid lineage in studies of mouse hematopoiesis [73], and is, to our knowledge, the only antibody specific for the extracellular domain of mouse GPA that is currently available. We report herein that infusion of this IgG GPA antibody caused development of anemia in mice deficient for the FcγR- or complement C3/C5-mediated mechanisms, and provide in vitro and in vivo evidence consistent with a non-classical mechanism of anemia likely involving RBC agglutination and subsequent splenic sequestration.

4.3 Materials and methods

4.3.1 Mice

C57BL/6, BALB/c, CD1 and DBA/2 mice were from Charles River Laboratories, FcR γ-chain-deficient mice (B6.129P2-FcεRIg<sup>tm1Rav</sup> N12) were from Taconic Farms, and C3-deficient mice (B6.129S4-C3tm1Crr/J) were from The Jackson Laboratory. Surgically splenectomized C57BL/6 mice and control C57BL/6 mice were from the Jackson Laboratory. All mice were used at 6-12 weeks of age. All animal experiments were approved by the Animal Care and Use Committee of St. Michael’s Hospital.
4.3.2 Reagents

Normal rat IgG and FITC-conjugated goat F(ab’)\(_2\) fragments of rat IgG-specific antibody was purchased from Caltag, and the anti-CD41 (MWReg30), anti-GPA (TER119), anti-CD24 (M1/69) antibodies, rat IgG2b κ isotype control (A95-1) antibodies, and the Annexin V-FITC apoptosis detection kit were from BD Biosciences.

4.3.3 Anemia

Anemia was induced in mice by intravenous injection of 45 μg of anti-GPA antibody TER119 in 200 μL PBS. At indicated times post injection, 10 μL of whole blood was drawn by saphenous bleeding and diluted 1: 100 in PBS/EDTA and another 1: 1,000 in PBS. The diluted blood (500 μL) was then acquired on a flow cytometer (Guava EasyCyte Mini System) for RBC counts. Detection of antibody binding was done as described [74]. Briefly, mouse blood was washed with PBS for 3 times (400g; 10 minutes), and incubated with FITC-conjugated goat F(ab’)\(_2\) anti-rat IgG at room temperature for 30 min in dark, then washed with PBS to remove unbound secondary antibody, and analyzed on a Guava EasyCyte Mini System.

4.3.4 Histopathology

Mouse spleen and liver tissues were obtained at autopsy, processed for histological examination, and stained with hematoxylin and eosin (H&E) as described [10]. Tissue slides were viewed with a Nikon Eclipse 80i microscope, and pictures were taken with a Nikon Digital Sight camera and acquired on Nis Element D3.00 SP5 software.
4.3.5  *In vitro RBC incubation with antibody*

Mouse blood was washed with PBS for 3 times (400g; 10 minutes), the buffy coat and plasma removed, and 200 μL was incubated with the indicated concentration of GPA antibody at 4°C, room temperature, or 37°C for 1 hour. Unbound antibody was washed away after the incubation. For detection of microagglutination, diluted RBC was observed on a hemocytometer with a Nikon Eclipse TS100 microscope. Pictures were taken with a Nikon Coolpix 4500 camera.

4.3.6  *Flow cytometric analysis of phosphatidylserine (PS) exposure*

The level of PS exposure on mouse erythrocytes was measured using an Annexin V-FITC assay on a Guava EasyCyte Mini System according to manufacturer’s instructions. Briefly, mouse blood was washed with cold PBS for 3 times (400g; 10 minutes), the buffy coat and plasma removed, and cells were resuspended in 1X Binding Buffer at 1 x 10^6 cells/mL. Add 5 μL of FITC Annexin V to 100 μL of the cell solution (1 x 10^5 cells) and incubate for 15 min at room temperature in dark. At the end of incubation, add 400 μL of 1X Binding Buffer and analyze by flow cytometry (Guava EasyCyte Mini System). Flow cytometric data were analyzed on FlowJo software (version X).

4.3.7  *Heat treatment of RBC*

Heat treatment of RBC was performed as described [75]. Briefly, whole blood was drawn by retro-orbital bleeding and diluted in citrate phosphage dextrose adenine (CPDA). Blood was washed 3 times with PBS (400g; 10 minutes), and buffy coat and plasma was removed. Dilute the washed blood in warm (50°C) PBS to 50% hematocrit (Hct), and incubated in 50°C water bath for 30 minutes. Then wash the cells 3 times with PBS (400g; 10 minutes).
4.3.8 Measurement of platelet counts

Platelet counts were determined as described [74]. Briefly, 10 μL of whole blood was drawn by saphenous bleeding and diluted 1:100 in PBS/EDTA. The diluted blood was centrifuged (170g; 2 minutes; brake off) to separate the platelet-rich plasma (PRP). The PRP was diluted 1:100 in isoton and platelet counts were determined on a Beckman Z2 Coulter Counter.

4.3.9 Statistical analysis

Data are presented as mean plus or minus standard error of the mean (SEM). Note, in some cases where the error bars are not apparent, this is due to a minimal SEM. Statistical analysis was performed with the Student t test using GraphPad Prism software. P<0.05 was considered significant.

4.4 Results

4.4.1 Induction of anemia by the GPA antibody

GPA antibody (45 μg/mouse) was found to bind efficiently to circulating RBC (Fig. 2), and cause increasing anemia with a nadir at 4 days post injection, followed by recovery in the next 4 days. Although the anti-GPA is a rat antibody, the response appeared to be RBC-specific as the isotype control did not cause changes in RBC counts (Fig. 3). An acute episode of hemoglobinuria was observed in the first day post-injection (Fig. 4), consistent with the possibility that some RBC could potentially be destroyed by intravascular hemolysis.
Figure 2. Sensitization of peripheral RBC with the anti-GPA antibody. C57BL/6 mice were injected with GPA antibody, and bled at the indicated times for anti-RBC antibody binding analysis. RBC from mice untreated (◊) or treated with GPA antibody (■) were stained with a fluorescent anti-rat IgG antibody, and analyzed by flow cytometry for mean fluorescence intensity (MFI). n=6 mice in total from 3 independent experiments.
Figure 3. Induction of anemia by the anti-GPA antibody. C57BL/6 mice were injected with GPA antibody, and bled at the indicated times for RBC enumeration. Blood from mice untreated (○), or treated with GPA antibody (□) or isotype control (●) were used to enumerate RBC by flow cytometry. n=6 mice in total from 3 independent experiments.
Figure 4. Induction of hemoglobinuria by the anti-GPA antibody. C57BL/6 mice were injected with GPA antibody. Upper panel: urine accumulated from mice untreated (left) or treated with GPA antibody (right) in the first day post-injection. Shown are typical pictures from 3 independent experiments. Lower panel: Urine samples from mice transfused with the GPA antibody displayed absorbance peaks at ~414 nm, confirming that the pigment was free hemoglobin [42].
4.4.2 Involvement of the spleen and liver in GPA antibody-mediated anemia

To determine the role of the spleen in anemia induced by the GPA antibody, mice were sacrificed at different times post injection. The antibody induced marked splenomegaly (Fig. 5A), and the increase in spleen weight was inversely related to the decrease in RBC counts over the first 4 days (Fig. 5B), suggesting that the spleen may be a target site for RBC sequestration and/or destruction. Histological analysis (Fig. 6) confirmed the accumulation of RBC in the spleen observable by 30 minutes post antibody injection. The red pulp was massively hemorrhaged, and red pulp expansion remained evident until 2 days post-injection. Interestingly, the fastest growth in splenic size, observed from day 2 to day 5 post-injection, was associated with white pulp rather than red pulp expansion, after which the normal tissue architecture was gradually regained. Conversely, hepatomegaly was not observed, and the most prominent liver pathology was consistent with necrosis of the parenchymal cells which was most severe on day 2 post-injection and subsided thereafter (Fig. 7).

To investigate whether the spleen was essential for this anemia, we injected surgically splenectomized mice with the GPA antibody. Interestingly, the development of anemia was partially but significantly delayed in the absence of a spleen. However, the splenectomized mice went on to develop a similar severity of anemia on day 4, and experienced delayed recovery of circulating RBC as compared to their normal littermates (Fig. 8).
Figure 5. *Induction of splenomegaly by the GPA antibody.* C57BL/6 mice were injected with GPA antibody, and sacrificed at the indicated times post-injection. (A) Representative images of spleens taken from untreated mice (Nil) and mice treated with the GPA antibody for 2 days (2d) or 5 days (5d). (B) Spleen weight (▲) and RBC counts (Δ). n=2 mice in total from 2 independent experiments.
Figure 6. Spleen pathology mediated by the GPA antibody. C57BL/6 mice were injected with GPA antibody, sacrificed at the indicated times post-injection, and spleen was harvested and processed for H&E staining. Shown are representative graphs from 3 independent experiments. Bars represent 0.5 mm.
Figure 7. Liver pathology mediated by the GPA antibody. C57BL/6 mice were injected with GPA antibody, sacrificed at the indicated times post-injection, and liver was harvested and processed for H&E staining. Shown are representative graphs from 3 independent experiments. Bars represent 0.5 mm.
Figure 8. Role of spleen in the development of anemia mediated by GPA antibody. Normal and surgically splenectomized mice were injected with the GPA antibody. Blood from untreated mice (normal: ●; splenectomized: ○) or mice treated with GPA antibody (normal: ■; splenectomized: □) were used to enumerate RBC by flow cytometry. n=3 mice in total from 3 independent experiments. The asterisks (**) P<0.01, (***) P<0.001) refer to statistical significance between normal (■) and splenectomized (□) mice treated with the GPA antibody.
4.4.3 *Lack of involvement of FcγR- and complement-mediated mechanisms in GPA antibody-induced anemia*

To determine if the anemic effect of the anti-GPA antibody involves FcγR- or complement-mediated mechanisms, we next compared the severity of anemia in Fcγ chain−/− (Fcγ−/−) and complement C3−/− mice. At the time of the maximal contribution of the spleen to anemia (1 day post-injection), the degree of RBC destruction in the Fcγ−/− or C3−/− mice was not found to be significantly different from C57BL/6 mice, as all mice experienced anemia to a similar degree 24 hours post injection. Because hemoglobinuria was still detected in the C3−/− mice (data not shown), we further evaluated the potential involvement of complement-mediated intravascular hemolysis using DBA/2 mice which are deficient in the complement C5 protein. These C5-deficient mice also developed a similar degree of RBC destruction as compared to C57BL/6 and BALB/c mice (Fig. 9).
Figure 9. GPA antibody-induced anemia in Fcγ<sup>-/-</sup>, C3<sup>-/-</sup>, and C5-deficient mice. C57BL/6, Fcγ<sup>-/-</sup>, C3<sup>-/-</sup>, DBA/2 (C5-deficient) and BALB/c mice were injected with the GPA antibody, and bled at 24 hours post-injection for RBC enumeration. RBC counts were expressed as a percentage of initial (baseline) counts. n=4 mice in total from 4 independent experiments.
4.4.4 GPA antibody does not induce increased phosphatidylserine exposure

Because of evidence in the literature suggesting an indispensible role of macrophages in the induction of anemia by the anti-GPA antibody [76], yet we did not find FcγR- or complement-mediated phagocytosis to be a major mechanism, we tested the possibility that the GPA-specific antibody induces phosphatidylserine (PS) exposure which then activates macrophage PS receptor-mediated phagocytosis, using a PS flow cytometry detection kit. RBC were drawn from mice injected with anti-GPA antibody 24 hours post injection, and did not seem to have increased PS expression, whereas heat-damaged RBC had a distinct population with increased PS expression (Fig. 10).
Figure 10. Detection of PS exposure on GPA antibody-treated RBC. Heat-damaged RBC were used as positive control. Shown are representative histograms from 2 independent experiments. (A) Compared to RBC from naive mice (shaded histogram), heat-damaged RBC (open histogram) exhibited a population of cells with enhanced PS exposure as measured by an Annexin V-FITC kit. (B-C) In contrast, RBC from mice treated with the GPA antibody for 30 min (B; open histogram) or 1 day (C; open histogram) did not seem to have increased PS expression compared to untreated RBC (shaded histogram).
4.4.5 GPA antibody-mediated RBC agglutination in vitro

Since neither FcγR- nor complement-mediated phagocytosis was found indispensable in RBC clearance caused by the GPA antibody, we tested the possibility that the antibody could cause direct damage to RBC. To eliminate phagocytic cells and complement, we removed the buffy coat and washed the RBC to remove all plasma, and then incubated these RBC with the GPA antibody at increasing concentrations in vitro for 1 hour. Flow cytometric RBC counting exhibited a dose-dependent reduction in RBC detected (Fig. 11). This reduction was not observed with non-specific rat IgG or with another rat-anti-mouse RBC antibody of the same IgG2b isotype (anti-CD24). Similar reductions in RBC counts were observed from RBC derived from C3⁻/⁻ mice (data not shown). Forward and side scatter identified an increase in 2 populations of events in association with this observed reduction in cell counts: one corresponding to higher forward and side scatter (larger cells/complexes) and the other consistent with cell debris (Fig. 12).

Bifunctional ligation of GPA has been reported previously by Brain et al [33] to initiate hemolysis associated with osmotic stress in an in vitro system. To similarly test if the in vitro RBC findings caused by the anti-GPA antibody here is affected by the pressure of ions, mouse RBC were incubated with the antibody in solutions with increasing proportions of isotonic sucrose to PBS (Fig. 13). Similar to the findings of Brain et al [33], complete replacement of PBS with sucrose abrogated the observed phenomenon at the end of the 1-hour incubation, while the partial replacement with sucrose had an intermediate effect.
To determine if the decreased RBC cell counting by flow cytometry could be due to RBC agglutination, samples were observed by microscopy (Fig. 14). Incubation with GPA antibody at room temperature was associated with a dose-dependent increase in the proportion of cells in agglutinated form. This antibody-associated agglutination was enhanced at 37°C, and delayed at 4°C. No agglutination was found with cells incubated with normal rat IgG (data not shown) or the anti-CD24 antibody at any temperature tested.
Figure 11. GPA antibody incubation with mouse RBC in vitro induces dose-dependent hemolysis. RBC from C57BL/6 mice were incubated with increasing concentration of the GPA antibody (■), anti-CD24 (▲), or normal rat IgG (○) in vitro for 1 hour, washed, and RBC counts determined on a flow cytometer. n=3 mice in total from 3 independent experiments.
Figure 12. Flow cytometry scatter plot of mouse RBC incubated with the GPA antibody.

RBC from C57BL/6 mice were incubated with the GPA antibody, anti-CD24, or normal rat IgG at the indicated concentrations in vitro for 1 hour, washed, and RBC acquired on a flow cytometer. Representative forward and side scatter dot-plot from 2 independent experiments were shown.
Figure 13. GPA antibody-induced *in vitro* hemolysis is dependent on presence of extracellular ions. RBC from C57BL/6 mice were incubated with increasing concentration of the GPA antibody in PBS (●), isotonic sucrose (□), or PBS/sucrose (ratio of volume=1:1; ◊) for 1 hour, washed, and RBC counts determined on a flow cytometer. n=2 mice in total from 2 independent experiments.
Figure 14. GPA antibody-induced RBC agglutination in vitro. RBC from C57BL/6 mice were incubated with the GPA antibody or anti-CD24 at the indicated concentrations and temperature for 1 hour. Shown are representative microscopic examination of cells post incubation from 2 independent experiments.
4.4.6  *GPA antibody-mediated RBC agglutination in vivo*

To determine if the GPA antibody-mediated anemia was attributable to agglutination *in vivo*, blood films were prepared with whole blood from mice injected with the antibody. Microagglutinates were clearly visible in mice 30 min post injection of GPA antibody, and persisted in these mice at 24 hours-post injection. Individual RBC appeared to have visible variation in sizes on the first 2 days post-injection, and polychromasia was observed at later stage of the anemia (Fig. 15).
Figure 15. GPA antibody-induced RBC agglutination in vivo. C57BL/6 mice were injected with the GPA antibody, and terminally bled at the indicated times post-injection for blood smears (H&E). Shown are typical smears from 3 independent experiments. Original magnification x400.
4.5 Discussion

Allo- and auto-antibodies to human glycophorin A (GPA)-associated antigens can mediate immune RBC destruction, leading to transfusion reactions, hemolytic disease of the newborn (HDN), and autoimmune hemolytic anemia (AHA) [77]. Anti-GPA antibodies encountered in AHA, albeit relatively rare, are frequently associated with severe hemolysis unexplained by routine serological findings [24]. As such, treatment remains experience-based and better understanding of the pathophysiology is needed [9, 78]. In the current study, we initiated work to model anti-GPA antibody-mediated anemia in mice using the monoclonal anti-mouse GPA antibody TER119 [36].

The GPA antibody caused rapid and severe anemia in mice, inducing an acute episode of hemoglobinuria and dropping peripheral RBC counts to less than 50% of baseline level within 2 days after injection. In an attempt to determine the mechanism(s) of RBC destruction and/or clearance, we first considered the two principle mechanisms of antibody-mediated hemolysis in AHA, namely extravascular hemolysis primarily executed by splenic macrophages and liver Kupffer cells and, more rarely, intravascular hemolysis as a result of complement-induced lysis [25]. Surprisingly, mice genetically deficient in the FcR γ chain, which are deficient in the functions of all 3 activating Fc receptors in mice (FcγRI, III and IV) [50, 79], were not protected from anemia caused by the anti-GPA antibody, and neither were mice genetically deficient in complement C3. Because hemoglobinuria was observed in both the C57BL/6 and C3−/− mice treated with the antibody, to rule out the possibility that intravascular hemolysis may have occurred via C3-independent C5 generation [80], we repeated the experiment with DBA/2 mice which are naturally deficient in C5 [81], and showed that similar degree of anemia persisted in the absence of C5. Although C4b (upstream from C3 in complement cascade) is also capable of
binding to macrophage complement receptor (CR) for initiation of phagocytosis [82], this seems unlikely because C4-deficient mice were not protected from anemia following injection with the same anti-GPA antibody (D. R. Branch, personal communication, 2011).

The lack of prominent involvement of FcR- or complement-dependent hemolysis was further supported by in vitro finding where in the absence of phagocytic cells and complement components, the anti-GPA antibody was still able to cause dose-dependent RBC damage attributable to cell aggregation. Temperature analysis revealed that the anti-GPA does not behave like a typical cold agglutinin; in fact, agglutination was more efficient at 37°C than room temperature, but prevented at 4°C, suggesting that the antibody is more consistent with a “warm agglutinin” to rapidly bind and agglutinate circulating RBC. In addition, unlike cold agglutinin-mediated hemolytic anemia in which the major mechanism of hemolysis is complement-dependent [83], our in vitro and in vivo results consistently showed that neither agglutination nor the associated hemoglobinuria required complement.

We next sought to determine if agglutination was contributing to the development of anemia in vivo. Peripheral blood smears revealed that microagglutinates formed as soon as 30 min following intravenous injection of the anti-GPA antibody. Subsequently, reduction in peripheral RBC counts was associated with splenomegaly due to massive accumulation of red cells mainly in the red pulp of the spleen. The GPA antibody may have pathogenic consequences beyond anemia. Red cell agglutination was accompanied by liver injury, which progressed to pronounced necrosis which may be secondary to reduced delivery of oxygen to hepatocytes as a consequence of the extensive agglutination. This had been described by reports where antibody-mediated RBC aggregates were thought to contribute to fatal anemia as a result of hepatic failure [84, 85]. Notably, antibodies specific for human GPA determinants were also found in some of
these reported patient cases [86]. In addition, we found that the antibody-mediated pathologies may have additional hemostatic effect. In particular, despite no binding to mouse platelet *in vitro* or *in vivo* (data not shown), we observed that, in addition to anemia, injection of the anti-mouse GPA was associated with a ~30-40% drop in peripheral platelet counts by 1 day post-injection (Appendix 1A), and like the anemia, this thrombocytopenic effect persisted in both the FcγR- and the complement C3-deficient mice (Appendix 1B). Interestingly, Ott and colleagues [69] recently reported that platelet-rich plasma (PRP) obtained from suspensions containing agglutinated human RBC had lower platelet counts, which they suggested was likely due to the avid binding of platelets and formation of RBC-platelet aggregates. Therefore one possibility consistent with our observation is that in the anti-GPA-treated mice, platelets could have been trapped in/attracted to the microagglutinates as they form rapidly in the blood stream, and thus resulting in transient thrombocytopenia. Alternatively, platelet pooling in an enlarged spleen is long known to contribute to thrombocytopenia in splenomegalic patients [87, 88]. Given the massively enlarged and congested spleen we observed in GPA antibody-treated mice, the accompanied thrombocytopenia could have been secondary to splenomegaly.

Of interest, the role of the spleen in the progression of anemia seems two-fold. Initially, it could serve as a primary target site for the filtering and sequestration of agglutinated RBC in the red pulp in the first 2 days post-injection. This period was when the blood smears revealed individual RBC with visibly variable sizes, suggesting that an adaptive volume regulation mechanism may have been triggered as a result of splenic entrapment [72]. Accordingly, consistent with previous reports showing correlation between spleen sequestration/clearance and response to splenectomy in patients [10, 89], we report a transient but significant effect of splenectomy in delaying the anemia at this stage. On the other hand, the most striking increment in spleen weight occurring between day 3 and day 5 was a result of massive white pulp expansion likely due to replacement
of previously hemorrhaged areas with mitotically active mononuclear cells. Data in splenectomized mice during the same period of time (day 3-8) revealed that the absence of spleen was associated with sustained anemia, supporting the interpretation that the spleen may be involved in facilitating the recovery stage of the anemia, which is consistent with the general belief that the murine spleen constitutes a major hematopoietic organ [90-92], especially in response to acute anemia [93]. Interestingly, a similar phenomenon was observed in all other RBC-specific antibodies tested (anti-CD24 and anti-band 3; Appendix 2), where we also report a delay in the recovery in splenectomized mice as compared to their normal littermates.

Multivalency-mediated hemagglutination, which requires the polymeric forms of antibodies [32], has been shown to be responsible for IgM- and IgA-mediated severe anemia both in mouse and human [10, 31]. However, IgG antibodies, as is the anti-GPA antibody TER119 used in the current study, are monomers seldom known to directly crosslink RBC in causing immune hemolytic anemia [6]. Instead, an indirect mechanism could have been afforded by the IgG TER119 by binding of GPA and thereby inducing changes in cell aggregability. Interestingly, GPA with its high sialic acid content is the major contributor to the negative cell surface charge believed to be responsible for the electrostatic repulsion that prevents RBC aggregation [36]. Reduction in the sialic acid, achieved by neuraminidase-mediated hydrolysis, for instance, has been shown to enhance RBC aggregation [69]. Similarly, the binding of GPA by the antibody may have rendered the RBC more prone to agglutination by inducing membrane destabilization. Support for this interpretation is provided by published studies of anti-human GPA cold agglutinin (CA) where the authors proposed a novel mechanism of alteration in membrane permeability to explain the severe hemolysis in absence of significant complement participation [9, 33, 34]. In the current study we also tested their hypothesis that osmotic stress is contributing to the antibody-mediated cell damage. By subjecting isolated mouse RBC to incubation with the
antibody in isotonic sucrose, we report delayed agglutination than if incubation was performed in the cation-loaded PBS. It should be noted that, despite the antibodies in the cited human studies being cold agglutinins, agglutination was minimized by the authors so as to demonstrate cation permeability as a novel mechanism of hemolysis. Our results in a mouse model, in addition to supporting their interpretation that antibody binding to GPA can affect membrane permeability, seemed to suggest that agglutination itself can also be influenced by the presence of cations.

Finally, despite a lack of evidence supporting a prominent involvement of FcγR- or complement-dependent phagocytosis, it is likely that phagocytes may still play a role in mediating GPA antibody-mediated anemia. Recently it was reported that liposomal clodronate, a potent antimacrophage agent, had efficacy in preventing anemia induced by the same anti-GPA antibody in mice [76]. In addition, pretreatment with an inhibitor of the syk protein tyrosine kinase, which is involved in signal transduction in phagocytes, was also found to be partially protective of this anti-GPA antibody-mediated anemia [94]. These observations suggest that macrophages might still contribute to elimination of antibody-targeted RBC [95]; though judging from our data, their involvement are likely subsequent/secondary to the rather rapid action of agglutination. This interpretation is further supported by a recently proposed two-phased RBC clearance mechanism in a mouse model of hemolytic transfusion reaction [96], where it was suggested that a first phase of sequestration of incompatible RBC was followed by a second phase of terminal clearance dependent on phagocytes.

In conclusion, using a mouse model of IHA, we showed that an IgG GPA-specific antibody caused severe anemia that persisted in both FcγR- and complement-deficient mice, and our in vitro and in vivo results support a novel mechanism of IgG-mediated RBC warm agglutination and subsequent sequestration and destruction in the spleen. Consistent with previous reports
showing a correlation between spleen sequestration and response to splenectomy in patients [10, 89], we reported a transient but significant effect of splenectomy in delaying the GPA antibody-induced anemia.
Chapter 5

Efficacy of IVIg Treatment in a Murine Model of Autoimmune Hemolytic Anemia
5 Efficacy of IVIg Treatment in a Murine Model of Autoimmune Hemolytic Anemia

5.1 Abstract

Intravenous immunoglobulin (IVIg) is used to effectively treat autoimmune diseases including immune thrombocytopenia (ITP), yet its use in the treatment of autoimmune hemolytic anemia (AHA) remains controversial due to inconsistent efficacy, with no established explanation as to why some patients respond while others do not. AHA is characterized by red blood cell (RBC) destruction by antibodies against RBC-specific antigens, and the current paradigm suggests that RBC clearance is primarily caused by macrophage/complement-mediated phagocytosis and/or lysis. In this study, we tested the efficacy of IVIg therapy in a murine model of immune hemolytic anemia (IHA) induced by 4 IgG mouse erythrocyte-specific antibodies: anti-band 3, -CD24, -GPA, and -Kell. We then evaluated the degree of RBC destruction attributable to FcγR- vs complement-dependent mechanisms using mice genetically deficient for the Fc receptor γ chain (Fcγ−/−) or complement C3 (C3−/−), respectively. Consistent with previous reports, we found a minimal role for complement-mediated mechanism in anemia caused by these antibodies. When injected with the anti-band 3 or anti-CD24 antibody, the Fcγ−/− mice were found to develop less than 50% of the anemia observed in C57BL/6 mice, suggesting a major role of FcγR-mediated RBC clearance mechanisms induced by these 2 antibodies. In contrast, only a minor degree of anemia caused by the anti-GPA was attributable to FcγR-dependent mechanism(s). Interestingly, the Fcγ−/− mice seemed to develop a similar degree of anemia in response to the anti-Kell antibody but have accelerated recovery as compared to C57BL/6 mice. When IVIg was infused into AHA mice, a significant response was observed in anti-band 3 and anti-CD24 antibody treated mice, but not in anti-GPA treated mice, and IVIg was also associated with
accelerated recovery in anti-Kell treated mice. In conclusion, similar to observations in human patients, our findings demonstrate a varied efficacy of high dose IVIg treatment in a murine model of IHA. With the use of 4 anti-mouse RBC antibodies we found that response to IVIg treatment was associated with the presence of FcγR-dependent mechanism(s) of anemia.

5.2 Introduction

Intravenous immunoglobulin (IVIg) is an immunoglobulin (Ig) G fraction prepared from large pools of plasma, typically from thousands of blood donors. Initially an antibody replacement therapy for patients with immune deficiency [97], and still is, IVIg was first reported to be an effective treatment for autoimmune diseases when Imbach et al [13] demonstrated in 1981 that high dose administration of IVIg was associated with a rapid reversal of thrombocytopenia in children. Discoveries of efficacy in other autoimmune conditions soon followed [98-101], and IVIg is currently FDA-approved for indications in immune thrombocytopenia (ITP) among other diseases, and also used off-label in numerous other disorders. One of the off-label uses is in the treatment of autoimmune hemolytic anemia (AHA), a relatively uncommon autoimmune disease characterized by autoantibodies that target and attack autologous red blood cell (RBC), leading to anemia which can be at times life-threatening [102, 103]. Treatment of AHA with IVIg was first attempted in the early 1980s in a series of studies usually involving less than 10 patients, and the results were inconsistent: limited studies reporting efficacy [16, 17, 104] were balanced by others showing no beneficial response [14, 15]. A mega-analysis was later published, reporting an overall response rate of 40% in 73 patients [105]. In addition, the authors were able to identify 1) a low pre-treatment hemoglobin level; and 2) presence of hepatomegaly to be
strongly associated with success of IVIg treatment. However, their results did not allow a firm conclusion on the impact of a specific RBC clearance mechanism on treatment efficacy. It was acknowledged that such questions require a controlled trial to answer, which is extremely hard due to the highly heterogeneous population of this relatively rare disease, and the fact that most patients were on multiple concurrent medications in all the IVIg-AHA studies cited.

Therefore, in the current study we took advantage of a murine model of passively induced immune hemolytic anemia (IHA) that allowed us to study the efficacy of IVIg therapy. Similar to observations in patients, we found a varied efficacy of IVIg in the treatment of mouse IHA with a panel of four monoclonal RBC-specific antibodies including an anti-band 3, an anti-CD24, an anti-GPA, and an anti-Kell. In an attempt to identify disease variables correlated with treatment success, we evaluated the degree of anemia caused by each antibody attributable to Fcγ receptor (FcγR)- versus complement C3-dependent mechanisms, and report a potential association between IVIg efficacy and FcγR-dependent anemia.

5.3 Materials and methods

5.3.1 Mice

C57BL/6 and CD1 mice were purchased from Charles River Laboratories, FcR γ-chain-deficient mice (B6.129P2-Fcer1g^tm1RavN12) were from Taconic Farms, and C3-deficient mice (B6.129S4-C3tm1Crr/J) were from The Jackson Laboratory. All mice were used at 6-10 weeks of age. All animal experiments were approved by the Animal Care and Use Committee of St. Michael’s Hospital.
5.3.2  **Reagents**

Normal rat IgG and FITC-conjugated goat F(ab’)2 fragments of rat IgG-specific antibody was purchased from Caltag Laboratories, FITC-conjugated goat F(ab’)2 anti-mouse IgG(H+L) was from Jackson ImmunoResearch Laboratories, the anti-CD41 (MWR30, IgG1), anti-GPA (TER119, IgG2b), and anti-CD24 (M1/69, IgG2b) antibodies were from BD Biosciences, the anti-band 3 (34-3C, IgG2a) was from Hycult Biotech, and anti-Kell antibody (MIMA87, IgG2a) was from Dr Gregory Halverson (New York Blood Center). IVIg was either Gamunex 10% (Talecris Biotherapeutics) or Privigen 10% (CSL Behring).

5.3.3  **Anemia**

Unless otherwise indicated, anemia was induced in mice by intravenous injection of 45 μg of anti-GPA antibody (TER119), 70 μg of anti-CD24 antibody (M1/69), 50 μg of anti-band 3 antibody (34-3C), or 50 μg of anti-Kell antibody (MIMA87) in 200 μL PBS. At indicated times post injection, 10 μL of whole blood was drawn by saphenous bleeding and diluted 1:100 in PBS/EDTA and another 1:1,000 in PBS. The diluted blood (500 μL) was then acquired on a flow cytometer (Guava EasyCyte Mini System) for RBC counts. Detection of antibody binding was done as described [74]. Briefly, mouse blood was washed with PBS for 3 times (400g; 10 minutes), and incubated with FITC-conjugated secondary antibody at room temperature for 30 min in dark, then washed with PBS to remove unbound secondary antibody, and analyzed on a Guava EasyCyte Mini System.
5.3.4 **IVIg treatment**

IVIg was infused into mice using a protocol modified from Pottier et al [106]. Briefly, the indicated groups of mice were treated with a total of 80 mg/mouse (generally equivalent to 4g/kg) IVIg by intravenous injection, including 40 mg/mouse (~2g/kg) injected 24 hours before injection of anti-RBC antibody, and another 2 injections of 20 mg/mouse (~1g/kg) at 1 day and 3 days post injection of anti-RBC antibody.

5.3.5 **Calculation of FcγR-dependent anemia and IVIg ameliorative effect**

Calculation was done as follows. FcγR-dependent anemia = 1- (RBC counts in nil Fcγ−/− mice - RBC counts in antibody treated Fcγ−/− mice)/(RBC counts in nil C57BL/6 mice - RBC counts in antibody treated C57BL/6 mice). IVIg ameliorative effect = 1- (RBC counts in nil C57BL/6 mice - RBC counts in antibody treated C57BL/6 mice with IVIg treatment)/(RBC counts in nil C57BL/6 mice - RBC counts in antibody treated C57BL/6 mice).

5.3.6 **Statistical analysis**

Data are presented as mean plus or minus standard error of the mean (SEM). Note, in some cases where the error bars are not apparent, this is due to a minimal SEM. Statistical analysis was performed with the Student t test using GraphPad Prism software. \( P < .05 \) was considered significant.
5.4 Results

5.4.1 IVIg efficacy in ameliorating RBC destruction is a mouse model of AHA

Reported efficacy of high dose IVIg therapy is inconsistent in the treatment of immune mediated anemia in AHA patients [105]. To investigate if IVIg can ameliorate anemia in a mouse model of AHA, C57BL/6 mice were injected with one of 4 monoclonal IgG anti-mouse RBC antibodies, and efficacy of IVIg was determined. All 4 antibodies induced a significant drop in RBC counts with a nadir reported on day 4 post injection (Fig. 16~19). With the anti-band 3 (Fig. 16), anti-CD24 (Fig. 17) or anti-GPA (Fig. 18) antibody, the peripheral RBC counts started to increase from day 4 onwards, whereas anemia caused by the anti-Kell antibody was characterized by sustained low RBC counts and no recovery until day 8 (Fig. 19).

IVIg therapy was administered over a five-day period on alternate days: first injection of 2g/kg was given 1 day before the anti-RBC antibody injection, and a second and a third injection of 1g/kg each after 1 and 3 days of antibody injection, respectively. The total dose of 4g/kg IVIg was associated with significant increase in RBC counts in anti-band 3 (Fig. 16) and anti-CD24 (Fig. 17) antibody treated mice, raising RBC counts on day 4 by 56% and 38%, respectively, suggesting a therapeutic effect of IVIg in ameliorating anemia caused by these 2 antibodies. In contrast, no increase in RBC counts was detected in the anti-GPA treated mice at any time (Fig. 18). Interestingly, IVIg had no effect in RBC counts in the “clearance phase” (day 0 to 4) in anti-Kell treated mice, yet was associated with accelerated recovery in the later phase of anemia (day 4 to 8; Fig. 19).
Figure 16. Efficacy of IVIg therapy for band 3 antibody-mediated anemia. C57BL/6 mice were injected with the band 3 antibody, and the indicated group also received treatment with a total of 4g/kg IVIg as described in “materials and methods”. Each arrow indicates an infusion of IVIg. Shown are peripheral RBC counts in untreated mice (○), and mice treated with anti-RBC antibody only (□) or IVIg + anti-RBC antibody (■). n=4 in total from 4 independent experiments. The asterisks (*P<.05, **P<.01) refer to statistical significance between antibody-induced anemia with (■) and without (□) IVIg treatment at the same time points.
Figure 17. Efficacy of IVIg therapy for CD24 antibody-mediated anemia. C57BL/6 mice were injected with the CD24 antibody, and the indicated group also received treatment with 4g/kg IVIg as described in “materials and methods”. Each arrow indicates an infusion of IVIg. Shown are peripheral RBC counts in untreated mice (○), and mice treated with anti-RBC antibody only (□) or IVIg + anti-RBC antibody (■). n=4 in total from 4 independent experiments. The asterisks (*P<0.05) refer to statistical significance between antibody-induced anemia with (■) and without (□) IVIg treatment at the same time points.
Figure 18. Efficacy of 4g/kg IVIg therapy for GPA antibody-mediated anemia. C57BL/6 mice were injected with the GPA antibody, and the indicated group also received treatment with 4g/kg IVIg as described in “materials and methods”. Each arrow indicates an infusion of IVIg. Shown are peripheral RBC counts in untreated mice (○), and mice treated with anti-RBC antibody only (□) or IVIg + anti-RBC antibody (■). n=4 in total from 4 independent experiments.
Figure 19. Efficacy of IVIg therapy for Kell antibody-mediated anemia. C57BL/6 mice were injected with the Kell antibody, and the indicated group also received treatment with 4g/kg IVIg as described in “materials and methods”. Each arrow indicates an infusion of IVIg. Shown are peripheral RBC counts in untreated mice (○), and mice treated with anti-RBC antibody only (□) or IVIg + anti-RBC antibody (■). n=4 in total from 4 independent experiments. The asterisks (**P<.01, *** P<.001) refer to statistical significance between antibody-induced anemia with (■) and without (□) IVIg treatment at the same time points.
5.4.2 *IVlg ameliorative effect of anemia is associated with FcγR-dependent mechanism*

In an attempt to characterize the mechanism of anemia caused by each anti-RBC antibody, we determined the degree of anemia attributable to FcγR-dependent versus complement-mediated mechanism(s) using mice genetically deficient for the Fcγ chain (Fcγ−/−) or complement component C3 (C3−/−), respectively (except for the anti-band 3 antibody, which was only tested in Fcγ−/− mice; however, this antibody has been tested in C3−/− mice in published works [107]).

A similar degree of anemia was found in C3−/− mice compared to C57BL/6 mice treated with anti-CD24 (Fig. 20A), anti-GPA (Fig. 20B), or anti-Kell (Fig. 20C) antibody. Although not tested in the current study, the anti-band 3 antibody at the same dose of 50 µg per mouse has been shown by Azeredo da Silveira and coworkers [107] to cause similar degree of anemia in C3−/− and C57BL/6 mice at the same dose used in this study (50 µg/mouse). Altogether, these results suggest a minimal role, if any, for complement-mediated mechanism in anemia caused by these antibodies.
**Figure 20. Anemia in complement C3-deficient mice.** C57BL/6 and C3⁻/⁻ mice were injected with the anti-CD24 (A), anti-GPA (B), or anti-Kell (C) antibody. Blood from untreated mice (C57BL/6: ○; C3⁻/⁻: □) and mice treated with anti-RBC antibody (C57BL/6: ●; C3⁻/⁻: ■) were used to enumerate RBC by flow cytometry. n=3 mice in total from 3 independent experiments.
On the other hand, when the Fcγ+/− mice were injected with the anti-band 3 (Fig. 21) and anti-CD24 (Fig. 22) antibody, the degree of anemia was considerably less than what was observed in the C57BL/6 mice on day 4, suggesting a role of FcγR-mediated mechanisms induced by these 2 antibodies. In contrast, in Fcγ+/− mice treated with the anti-GPA antibody, only a minor proportion of anemia was prevented as compared to the C57BL/6 mice (Fig. 23). Interestingly, following injection with the anti-Kell antibody, the Fcγ+/− mice seemed to develop similar degree of anemia as in the C57BL/6 mice, but have accelerated recovery in the period between day 4 and day 8 post-injection (Fig. 24). In addition, anti-Kell-treated Fcγ+/− mice were found no longer respond to IVIg treatment (data not shown).
Figure 21. Band 3 antibody-mediated anemia in FcγR-deficient mice. C57BL/6 and FcγR−/− mice were injected with the band 3 antibody. Blood from untreated mice (C57BL/6: ●; FcγR−/−: ■) and mice treated with antibody (C57BL/6: ○; C3−/−: □) were used to enumerate RBC by flow cytometry. n=6 mice in total from 3 independent experiments. The asterisks (**P<.01, ***P<.001) refer to statistical significance between C57BL/6 (○) and FcγR−/− (□) mice treated with the antibody.
Figure 22. CD24 antibody-mediated anemia in FcγR-deficient mice. C57BL/6 and Fcγ⁻/⁻ mice were injected with the CD24 antibody. Blood from untreated mice (C57BL/6: ●; Fcγ⁻/⁻: ■) and mice treated with antibody (C57BL/6: ○; C3⁻/⁻: □) were used to enumerate RBC by flow cytometry. n=6 mice in total from 3 independent experiments. The asterisks (***P<.01) refer to statistical significance between C57BL/6 (○) and Fcγ⁻/⁻ (□) mice treated with the antibody.
Figure 23. GPA antibody-mediated anemia in FcγR-deficient mice. C57BL/6 and Fcγ⁻ mice were injected with the GPA antibody. Blood from untreated mice (C57BL/6: ●; Fcγ⁻: ■) and mice treated with antibody (C57BL/6: ○; C3⁻: □) were used to enumerate RBC by flow cytometry. n=6 mice in total from 3 independent experiments. The asterisks (**P<.01, ***P<.001) refer to statistical significance between C57BL/6 (○) and Fcγ⁻ (□) mice treated with the antibody.
**Figure 24. Kell antibody-mediated anemia in FcγR-deficient mice.** C57BL/6 and Fcγ<sup>−/−</sup> mice were injected with the Kell antibody. Blood from untreated mice (C57BL/6: ●; Fcγ<sup>−/−</sup>: ■) and mice treated with anti-RBC antibody (C57BL/6: ○; C3<sup>−/−</sup>: □) were used to enumerate RBC by flow cytometry. n=6 mice in total from 3 independent experiments. The asterisks (*P<.05, **P<.01) refer to statistical significance between C57BL/6 (○) and Fcγ<sup>−/−</sup> (□) mice treated with the antibody.
As all 4 antibodies caused anemia with a nadir at 4 days post-injection, it was found that the FcγR-dependent anemia on day 4 was correlated with the relative efficacy of IVIg treatment on the same day (Table 2; upper panel). In addition, because with the anti-Kell antibody, the most significant effect of IVIg was the acceleration of recovery (day 4 to day 8), we observed that the FcγR-dependent anemia on day 8 was also correlated with significant ameliorative effect of IVIg at this time (Table 2; lower panel).
Table 5-1. Response to IVIg was associated with FcγR-dependent anemia. *P<.05, **P<.01, *** P<.001; ns= not significant.

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>FcγR-dependent anemia on day 4</th>
<th>IVIg ameliorative effect on day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 3</td>
<td>61%***</td>
<td>56%**</td>
</tr>
<tr>
<td>CD24</td>
<td>54%***</td>
<td>38%*</td>
</tr>
<tr>
<td>GPA</td>
<td>21%***</td>
<td>9% ns</td>
</tr>
<tr>
<td>Kell</td>
<td>37%*</td>
<td>19% ns</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>FcγR-dependent anemia on day 8</th>
<th>IVIg-ameliorative effect on day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kell</td>
<td>70%**</td>
<td>62%**</td>
</tr>
</tbody>
</table>
5.5 Discussion

Treatment for patients with AHA remains a challenging task as the current therapeutic regime is still essentially experience-based [11]. Despite its established efficacy in other autoimmune cytopenias (e.g., immune thrombocytopenia and autoimmune neutropenia) and low risk of side effects compared to other mainstay therapies of AHA (e.g., glucocorticoids and cytotoxic drugs), IVIg remains an off-label indication for the treatment of AHA due in part to inconsistent responses [105], with no established explanation as to why some patients respond but others do not. In the present study, we examined the mechanism of anemia as a potential factor in determining the efficacy of IVIg in a murine model of passively induced immune mediated hemolytic anemia (IHA).

Of the 4 IgG anti-mouse RBC antibodies used, three recognize RBC antigens that are of relevance to the human disease (band 3, GPA and Kell), but CD24 is not expressed on human erythrocytes [108]. All 4 antibodies induced significant anemia in mice, although two of the antibodies (anti-CD24 and anti-GPA) are rat antibodies, the response appears to be RBC specific as normal rat IgG does not cause changes in RBC counts (data not shown).

Similar to observations in human patients, our findings demonstrated differential efficacy of high dose IVIg treatment in the IHA mice. In the “clearance phase” of anemia, which is the period from the injection of the anti-RBC antibody to when the anemia reaches its nadir (day 0 to day 4), treatment with IVIg (4g/kg) was associated with significantly less anemia in mice injected with the anti-band 3 and anti-CD24 antibody. In contrast, whereas no significant efficacy of IVIg was detected in the clearance phase in anti-Kell antibody-treated mice, IVIg therapy was associated with accelerated recovery in peripheral RBC counts, indicating a likely role of IVIg in
facilitating this recovery phase of anti-Kell-mediated anemia. Taken together, these results suggested that not only is the efficacy of IVIg different in anemia caused by different antibodies, the underlying therapeutic mechanism could also be different.

Consistent with previous reports [31, 109], our results suggested a minor role, if any, of complement-mediated mechanisms in anemia mediated by these mouse erythrocyte-specific antibodies. As such, our data could not provide information as to the impact of IVIg treatment on complement actions, yet suggested that therapeutic agents targeting complement may not be effective in treating primary IHA in these mice.

In contrast, a significant component of FcγR-dependent anemia was identified 1) in the clearance phase in anti-CD24 and anti-band 3 treated mice, and 2) in the recovery phase of anti-Kell treated mice. Interestingly, in both cases, IVIg efficacy was found associated with FcγR-dependent anemia. Similar associations have been identified in a mouse model of ITP [110, 111] where it was showed that the anti-GPIbα antibodies which mediate predominantly Fc-independent thrombocytopenia, responded poorly to IVIg, whereas anti-GPIIbIIIa antibodies that cause Fc-dependent disease responded well.

Interestingly, in anemia caused by each antibody the initial/acute decrease in peripheral RBC counts (day 0 to day 2) seemed to be unaccounted for by the 2 mechanisms examined, despite the current understanding that antibody-mediated anemia is caused by FcR-mediated phagocytosis or complement-mediated phagocytosis and/or lysis, or a combination of both [24, 112]. The observations with the anti-GPA antibody were particularly striking, as this antibody was capable of causing severe anemia in the absence of the FcγR or complement C3. Further investigation of the mechanisms mediated by this antibody was presented in Chapter 4. Consistent with our observed correlation between presence of FcγR-dependent anemia and response to IVIg therapy,
we did not find IVIg to improve RBC counts in C57BL/6 mice treated with this antibody, but had an interesting observation in CD1 mice, where 50 μg of this anti-GPA antibody was associated with 75% death within 1 day of injection. Surprisingly, despite no major improvement in RBC counts, IVIg pretreatment was associated with 100% survival (Appendix 3). IVIg therapy has been shown to be associated with prevention of death in other disease mouse models. Chow et al [113] showed in an active model of ITP that bleeding mortality caused by antibody-mediated thrombocytopenia was preventable with IVIg treatment. More recently, Semple et al [114] demonstrated that IVIg prevents death in a mouse model of antibody-mediated transfusion-related acute lung injury (TRALI). Interestingly, in both studies, as well as our observation, IVIg was shown to prevent antibody-mediated death, despite different target of the antibody in each case. Whether the life-saving property of IVIg in all 3 studies is mediated by a common mechanism is a subject of future studies.

Another interesting antibody is the anti-Kell antibody. Similar to the other 3 antibodies, this anti-Kell antibody caused increasing anemia with a nadir at 4 days post-injection, yet the recovery was significantly delayed compared to anemia caused by the other antibodies. This could be due to longer half-life of antibody-coated RBC, yet we found, if anything, the anti-Kell antibody was associated with less antibody-positive circulating RBC, and the amount of bound antibodies was also considerably lower than anti-GPA and anti-band 3 (Appendix 4~6; note the difference in the scale of the y-axis). Anti-Kell antibodies are known as the second most common cause of severe hemolytic diseases of the newborn (HDN), second only to HDN caused by anti-D. Affected Kell-alloimmunized fetuses usually present with lower numbers of circulating reticulocytes and normoblasts, compared with anti-D-affected fetuses, despite elevated level of erythropoietin released [38]. Vaughan et al [39] demonstrated that monoclonal IgG and IgM antibodies specifically inhibited growth of Kell-positive (but not Kell-negative) erythroid progenitor cells in
a dose-dependent manner, whereas anti-D had no such effect. Their findings were furthered by Daniels et al [40] who showed that Kell antigen appears on erythroid progenitor cells early in erythropoiesis, prior to the expression of GPA and band 3. The authors went on to show that Kell-positive progenitor cells sensitized with anti-Kell sera from pregnant women elicited a strong response from monocytes in vitro, and proposed that anti-Kell but not anti-D may cause fetal anemia by promoting immune destruction of the Kell-positive erythroid early progenitor cells by macrophages in the fetal liver.

Whether our results showing delayed recovery in anti-Kell antibody-mediated anemia are due to a similar mechanism, namely inhibition of erythropoiesis by anti-Kell-mediated immune destruction of Kell-expressing erythroid progenitor cells, remains to be explored in future studies. Nevertheless, such a mechanism is not only consistent with the observed delay in recovery, but may also explain the fact that in FcγR−/− mice this inhibition is reversed, consistent with the possibility that FcγR−/− mice could not mediate macrophage FcγR-dependent phagocytosis of these progenitor cells.

In conclusion, our results demonstrate that efficacy of IVIg in ameliorating IHA varies in relation to the mechanism of anemia in mice, with FcγR-mediated anemia being highly related to response to IVIg.
Chapter 6

Monoclonal Anti-RBC Antibodies Can Ameliorate Thrombocytopenia in a Murine Model of Passive ITP
6 Monoclonal anti-RBC antibodies can ameliorate thrombocytopenia in a murine model of passive ITP

6.1 Summary

To explore the potential for monoclonal erythrocyte-specific antibodies as an alternative treatment for immune thrombocytopenia (ITP) and to explore the mechanism of their therapeutic action, we tested a panel of four monoclonal anti-mouse RBC antibodies in a murine model of ITP. We report that three of these antibodies: one anti-glycophorin A (GPA), one anti-band 3 and one anti-Kell antibody, were capable of ameliorating ITP to a similar extent as intravenous immunoglobulin (IVIg) but at a potentially 3-log fold lower dosage as compared to IVIg. Improvement in peripheral platelet counts was found to be positively associated with the severity of anemia in mice injected with these RBC-specific antibodies. However, whether these anti-RBC antibodies mediate the amelioration via Fcγ receptor (FcγR) blockade remains unclear.

6.2 Introduction

The beneficial effect of IVIg in ameliorating thrombocytopenia in ITP was first discovered by Imbach et al [13] in 1981. Subsequent investigations in the early 80s [12, 115] suggested the most likely mechanism of its therapeutic action to be a transitory blockade of the reticuloendothelial system (RES). Salama et al [116] proposed that it is the IgG-coated autologous RBC rather than IgG themselves that mediate the interaction with the Fc receptors (FcR) on macrophages of the RES. Supporting Salama et al’s proposed mechanism, polyclonal anti-D was later demonstrated to reverse thrombocytopenia in D-positive but not D-negative patients [117, 118]; and polyclonal anti-D remains one of the first-line treatment for ITP in D-positive patients to this date [61]. Because polyclonal anti-D is in limited supply and priority of
use is given to treating the hemolytic disease of the newborn (HDN) in D-negative women, a small prospective study was undertaken to evaluate the therapeutic effect of monoclonal anti-D as an alternative to the polyclonal antibody, yet revealed a lack of beneficial response in 6 out of 7 ITP patients involved [62], making it unethical for further human studies with other monoclonal anti-D.

However, the ineffectiveness of monoclonal anti-RBC antibodies in treating ITP was later challenged by reports showing efficacy of some anti-mouse RBC antibodies in ameliorating thrombocytopenia in a mouse model of passively induced ITP. Song et al [63] studied a list of monoclonal anti-mouse RBC antibodies and found that two of them: one anti-GPA antibody (TER119) and one anti-CD24 antibody (M1/69) can protect against ITP induced by a monoclonal anti-platelet antibody (MWReg30). Hemolysis was observed with both anti-RBC antibodies, and the GPA-specific antibody appears to be more potent in RBC clearance. Song et al [64] in a subsequent study furthered their findings and showed that these monoclonal anti-RBC antibodies might mediate their therapeutic actions differently than IVIg.

Interestingly, the protective status of this anti-GPA antibody was later challenged by Aubin et al [66] although the efficacy of the anti-CD24 antibody was confirmed by the same authors. Difference in experimental protocol (notably delivery method of the anti-RBC antibody and strain of mice used) was mentioned to be likely contributor to this discrepancy, but the authors also pointed out their observation of moderate thrombocytopenia induced by the GPA antibody, when injected alone, suggesting that such observation is difficult to reconcile with a therapeutic effect. Nevertheless, a dose-dependent efficacy of the same GPA antibody was subsequently demonstrated by Deng and Balthasar [65] as an effective treatment of a mouse model of chronic
passive ITP. Interestingly, Deng and Balthasar did not use or mention the anti-CD24 antibody, despite recognizing anti-GPA antibody-associated severe hemolysis.

In this thesis, in addition to anti-GPA and anti-CD24, another 2 monoclonal IgG mouse erythrocyte-specific antibodies were characterized in terms of therapeutic efficacy in murine ITP in relation to their ability to cause RBC clearance. We report herein that 3 out of 4 of these antibodies were capable of ameliorating ITP to a similar extent as 2.5g/kg (ie., 50 mg/mouse) IVIg, although at much lower dosage. The ability to improve platelet counts seemed to associate with the extent of RBC clearance, but association of efficacy with the presence of FcγR-dependent RBC clearance mechanism, as previously asserted, was not supported. In addition, we also observed the thrombocytopenic effect of the GPA antibody, which we did not find to interfere with the ameliorative effect of this antibody in the amelioration of ITP.

6.3 Materials and methods

6.3.1 Mice

C57BL/6 mice were purchased from Charles River Laboratories, and FcR γ-chain-deficient mice (B6.129P2-Fcer1g<sup>tm1Rav</sup> N12) were from Taconic Farms. All mice were used at 6-10 weeks of age. All animal experiments were approved by the Animal Care and Use Committee of St. Michael’s Hospital.

6.3.2 Reagents

The anti-CD41 (MWReg30, IgG1, 2 µg/mouse), anti-GPA (TER119, IgG2b, 45 µg/mouse), and anti-CD24 (M1/69, IgG2b, 70 µg/mouse) antibodies were purchased from BD Biosciences, the anti-band 3 (34-3C, IgG2a, 50 µg/mouse) antibody was from Hycult Biotech, and the anti-Kell
(MIMA87, IgG2a, 50 µg/mouse) was from Dr Gregory Halverson (New York Blood Center). IVIg was either Gamunex 10% (Talecris Biotherapeutics) or Privigen 10% (CSL Behring), and used at 50 mg/mouse (equivalent to ~2.5g/kg).

6.3.3 **ITP and measurement of platelet counts**

Thrombocytopenia was induced in mice by intravenous injection of 2 µg of MWReg30 antibody in 200 µL PBS. Platelet counts were determined as described [74]. Briefly, 10 µL of whole blood was drawn by saphenous bleeding and diluted 1:100 in PBS/EDTA. The diluted blood was centrifuged (170g; 2 minutes; brake off) to separate the platelet-rich plasma (PRP). The PRP was diluted 1:100 in isoton and platelet counts were determined on a Beckman Z2 Coulter Counter.

6.3.4 **IVIg or RBC antibody treatment of ITP**

Pretreatment with IVIg or one of the monoclonal anti-RBC antibodies was given 24 hours prior to the induction of ITP with the MWReg30 antibody. Platelet counts were determined 24 hours post MWReg30 injection.

6.3.5 **Statistical analysis**

Data are presented as mean plus or minus standard error of the mean (SEM). Note, in some cases where the error bars are not apparent, this is due to a minimal SEM. Statistical analysis was performed with the Student t test using GraphPad Prism software. $P<.05$ was considered significant.
6.4 Results and discussion

6.4.1 Monoclonal anti-RBC antibodies can ameliorate murine ITP

To determine their efficacy in the amelioration of ITP, each of the 4 anti-RBC antibodies: anti-GPA (TER119), anti-CD24 (M1/69), anti-band 3 (34-3C) and anti-Kell (MIMA87) was injected intravenously into mice 24 hours before the induction of ITP by the anti-CD41 antibody MWReg30. All but M1/69 was associated with improved platelet counts (Fig. 25~28). In particular, the anti-GPA (45 µg/mouse; Fig. 25) and anti-band 3 (50 µg/mouse; Fig. 26) were found to achieve a similar increase in platelet counts as 50 mg IVIg, and 50 µg anti-Kell (Fig. 27) also delivered comparable efficacy as compared to 50 mg IVIg. Unexpectedly, 50 µg of the anti-CD24 antibody, which was originally shown by Song et al [63] to be effective, failed to affect platelet counts (data not shown), and even an increased dose of 70 µg seemed to still be ineffective (Fig. 28).
Figure 25. Efficacy of the GPA antibody in the amelioration of a murine model of passive ITP. C57BL/6 mice were injected with the GPA antibody (45 μg) or IVIg (50 mg). 24 hours later, mice were injected with 2 μg of the anti-platelet antibody MWRReg30 to induce thrombocytopenia. Mice were bled after a further 24 hours, and the platelet counts were determined. n=4 mice in total from 2 independent experiments. The asterisks (**P<.01, ***P<.001) refer to statistical significance between ITP mice with and without treatment.
Figure 26. Efficacy of the band 3 antibody in the amelioration of a murine model of passive ITP. C57BL/6 mice were injected with the band 3 antibody (50 µg) or IVIg (50 mg). 24 hours later, mice were injected with 2 µg of the anti-platelet antibody MWReg30 to induce thrombocytopenia. Mice were bled after a further 24 hours, and the platelet counts were determined. n=4 mice in total from 2 independent experiments. The asterisks (**P<.01, ***P<.001) refer to statistical significance between ITP mice with and without treatment.
Figure 27. Efficacy of the Kell antibody in the amelioration of a murine model of passive ITP. C57BL/6 mice were injected with the Kell antibody (50 µg) or IVIg (50 mg). 24 hours later, mice were injected with 2 µg of the anti-platelet antibody MWReg30 to induce thrombocytopenia. Mice were bled after a further 24 hours, and the platelet counts were determined. n=4 mice in total from 2 independent experiments. The asterisks (***P<.001) refer to statistical significance between ITP mice with and without treatment.
Figure 28. Efficacy of the CD24 antibody in the amelioration of a murine model of passive ITP. C57BL/6 mice were injected with the CD24 antibody (70 µg) or IVIg (50 mg). 24 hours later, mice were injected with 2 µg of the anti-platelet antibody MWReg30 to induce thrombocytopenia. Mice were bled after a further 24 hours, and the platelet counts were determined. n=4 mice in total from 2 independent experiments. The asterisks (**) refer to statistical significance between ITP mice with and without treatment.
To evaluate if degree of RBC clearance caused by each anti-RBC antibody correlate with the ability to correct platelet counts following induction of ITP, we compared the % increase in platelet counts and the % decrease in RBC counts caused by the same anti-RBC antibody on the corresponding day (ie., 24 hours after injection of anti-platelet antibody, which corresponds to 48 hours after injection of the anti-RBC antibody). A significant drop in RBC counts seemed to associate with a better ameliorative effect in ITP (Table 3). In fact, as the anti-CD24 failed to raise platelet count, it also induced the least degree of anemia.
Table 3. Therapeutic effect of the anti-RBC antibodies in ITP in relation to the RBC clearance they induce. Max. ↓ % RBC was measured on day 4 post anti-RBC antibody injection (see chapter 5).

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>↑% PLT</th>
<th>Corresp.↓ % RBC</th>
<th>Max. ↓% RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPA</td>
<td>50%</td>
<td>49%</td>
<td>57%</td>
</tr>
<tr>
<td>CD24</td>
<td>0</td>
<td>15%</td>
<td>28%</td>
</tr>
<tr>
<td>Band 3</td>
<td>57%</td>
<td>27%</td>
<td>51%</td>
</tr>
<tr>
<td>Kell</td>
<td>46%</td>
<td>29%</td>
<td>35%</td>
</tr>
</tbody>
</table>
6.4.2 **FcγR is required for MWReg30 to induce platelet clearance, but not required for the anti-RBC antibodies to mediate RBC clearance at the time of ameliorative effect**

One of the most prominent theories of the therapeutic action of anti-D in the treatment of D-positive ITP patients is the binding of anti-D coated RBC to the macrophage FcγR [119], thereby preventing binding of antibody-coated platelet by a competitive mechanism. We first determined if the development of ITP in our mouse model requires FcγR-mediated platelet clearance with the use of Fcγ chain knock-out (Fcγ−/−) mice, which are deficient in the functions of all 3 classes of activating Fc receptors (FcγI, III and IV). The Fcγ−/− mice were fully protected from thrombocytopenia induced by MWReg30 (Fig. 29), demonstrating that MWReg30 requires the FcγR to mediate platelet clearance.

As the Fcγ−/− mice do not develop any thrombocytopenia due to MWReg30, it is difficult to directly determine if FcγR is required for the anti-RBC antibodies to ameliorate ITP in this mouse model. We therefore examined RBC clearance in Fcγ−/− mice, instead, as an alternative measure of the engagement of FcγR by the anti-RBC antibodies. We found that, none of the 3 effective antibodies (anti-GPA, anti-band 3 and anti-Kell) seemed to require the FcγR in mediating anemia in the first 48 hours after injection of the anti-RBC antibody (Fig. 30), suggesting that the RBC clearance responsible for the attenuation of ITP was largely independent of FcγR-dependent mechanisms.
Figure 29. Fcγ<sup>-/-</sup> mice were fully protected from thrombocytopenia induced by MWReg30. C57BL/6 and Fcγ<sup>-/-</sup> mice were injected with 2 µg/mouse MWReg30 antibody, and bled 24 hours later to determine platelet counts. n=2 mice in total from 2 independent experiments. The asterisks (*P<.05) refer to statistical significance between nil and Fcγ<sup>-/-</sup> mice treated with MWReg30.
Figure 30. Degree of anemia in Fcγ−/− mice. C57BL/6 and Fcγ−/− mice were injected with the indicated anti-RBC antibody (anti-GPA: 45 μg; anti-CD24: 70 μg; anti-band 3: 50 μg; or anti-Kell: 50 μg), and bled 48 hours later to determine RBC counts. n=4 mice in total from 4 independent experiments. The dotted line indicates normal RBC counts in naïve mice.
Taken together, these results suggested that RBC clearance but not necessarily FcγR-mediated RBC clearance is associated with the ameliorative effect of monoclonal anti-RBC antibodies in a mouse model of ITP. It is likely that the antibody-coated RBC still compete with antibody-coated platelet for phagocytosis, but via different receptor/mechanism for the engagement of the macrophage.

6.4.3 **TER119 prevents MWReg30-induced thrombocytopenia but also mediates thrombocytopenia on its own**

Aubin et al [66] noticed decrease in platelet counts in anti-GPA (TER119)-treated BALB/c mice, which, in their argument, complemented their data showing anti-GPA as “unprotective” in mouse ITP induced by MWReg30. We also observed a transient thrombocytopenic effect of anti-GPA in C57BL/6 and also the Fcγ−/− mice (Appendix 1). The GPA antibody injected alone caused 30-40% drop in platelet counts by 24 hours post injection; after 24 hours, platelet counts started to recover. Interestingly, if mice received MWReg30 24 hours after they received anti-GPA, MWReg30 failed to cause any further decrease in platelet counts than what was already induced by anti-GPA (Fig. 31). In other words, because anti-GPA caused less thrombocytopenia than MWReg30, its thrombocytopenic effect did not interfere with its anti-thrombocytopenic effect.

It is not clear how anti-GPA mediates its thrombocytopenic effect; a more detailed discussion can be found in Chapter 4. Our data seem to argue against the assertion of Aubin et al [66] by showing that the seemingly paradoxical observations of pro- and anti-thrombocytopenic effects can be reconciled. In fact, one of the possible mechanisms of therapeutic action of anti-GPA in anti-platelet antibody-mediated ITP could be related to its thrombocytopenic action. As demonstrated by Deng and Balthasar [65] in their chronic ITP model, therapeutic dose of anti-GPA (50 µg in their case) was able to raise platelet counts to >200% of the initial platelet counts, that is, >100% more than normal level. We also observed a “rebound” in platelet level 3-4 days
after anti-GPA injection (data not shown) although not as pronounced. This “rebound” in platelet counts could potentially be due to compensatory action of the body’s hematopoietic system in response to platelet (and RBC) clearance induced by anti-GPA. Further studies will help to determine if a stimulation of thrombopoiesis plays a role in the therapeutic action of the GPA antibody in murine ITP.
Figure 31. Effect of anti-GPA on platelet counts. C57BL/6 mice were injected with 45 μg anti-GPA on day 0. 24 hours later, the indicated group also received 2 μg of the anti-platelet antibody MWReg30. Blood from mice treated with anti-GPA only (□) or anti-GPA+MWReg30 (♦) were used to enumerate platelet counts on the indicated times. The dotted line indicates platelet counts in mice treated with 2 μg MWReg30 only. n=2 mice in total from 2 independent experiments.
In conclusion, our results expanded the list of monoclonal anti-RBC antibodies that have an “anti-D-like” effect in ameliorating thrombocytopenia in a mouse model of ITP. We showed that an anti-band 3 and an anti-Kell, as well as the anti-GPA antibody previously identified by Song et al [63], can prevent ITP to a similar extent as IVIg, but at a potentially 3-log fold lower dosage as compared to IVIg. All 3 antibodies recognize “public” antigens expressed by almost all human RBCs, and therefore could potentially be of advantage in the treatment of D-negative ITP patients. Using FcγR− mice, we demonstrated that improvement of platelet counts coincided with significant RBC clearance that appeared to be largely FcγR-independent, despite a complete dependency on FcγR for development of thrombocytopenia by the platelet-specific antibody MWReg30, arguing against a therapeutic mechanism of competition for FcγR binding. However, it should be noted that these monoclonal anti-mouse RBC antibodies could behave very differently from polyclonal anti-D in human patients. An example is offered by the anti-GPA antibody: its ability to cause thrombocytopenia and ameliorate MWReg30-mediated thrombocytopenia at the same time seemed paradoxical, but might in fact suggest a novel therapeutic mechanism yet to be explored.
Chapter 7

Discussion
7 Discussion

7.1 Summary

Antibodies against erythrocyte-specific antigens are the focus of this thesis. A murine model of passively induced autoimmune hemolytic anemia (AHA) was optimized for a panel of mouse RBC-specific antibodies, and the mechanism(s) of RBC clearance were investigated. Data in this thesis from the use of four different antibodies consistently showed that mechanisms in addition to the classical macrophage Fc receptor (FcR)/complement-mediated extra- and intravascular hemolysis may be contributing to the severe anemia observed in mice (Chapter 5). Many factors can influence the pathogenicity of antibodies, including the antibody class, subclass, thermal amplitude, complement/macrophage activating efficiency and so on [112], and results presented in this thesis suggest that the antibody specificity may potentially be of importance. For instance, the glycophorin A (GPA)-specific antibody-mediated RBC agglutination is unexpected for an IgG antibody [6], but consistent with the role GPA plays in minimizing cell-cell interaction [36] (Chapter 4). Moreover, the Kell-specific antibody-induced Fc-dependent inhibition of recovery is also potentially related to the early expression of the Kell antigens on the erythroid precursor cells [40] (Chapter 5). Nevertheless, the canonical mechanism of extravascular hemolysis was well represented in this model, as two out of four of the antibodies (anti-band 3 and anti-CD24) were found to mediate primarily an FcR-dependent mechanism of clearance (Chapter 5).

The treatment of AHA continues to pose a clinical problem [11], and the current treatment regime remains experience-based as controlled clinical trials are extremely difficult due to the low numbers of patients and high frequency of underlying secondary conditions [6, 11]. The murine model therefore provides a platform for the evaluation and optimization of both known
and novel treatment regimes. In this thesis the efficacy of IVIg therapy, a well established treatment for many autoimmune conditions (but with inconsistent efficacy in AHA patients), was evaluated in murine AHA caused by the four mouse RBC-specific antibodies, and response to treatment was found to correlate with FcγR-dependent anemia (Chapter 5). A similar correlation of IVIg effect and FcγR-mediated cytopenia has been previously demonstrated in a murine model of immune thrombocytopenia [110, 111], and this thesis, to our best knowledge, is the first to demonstrate such a relationship in the treatment of AHA. In addition, a potential treatment with CD44 antibodies was also evaluated in the murine model of AHA. An effective treatment of FcγR-mediated murine ITP [63, 74], the anti-CD44 antibody KM114 was hypothesized to be an alternative treatment for FcγR-mediated anemia in the current mouse model, but was found ineffective in the amelioration of band 3 antibody-mediated anemia (Appendix 10).

In addition to the elucidation of the pathophysiological effects of the RBC-specific antibodies, this thesis also explored the therapeutic potential of these monoclonal antibodies in the treatment of murine ITP analogous to the use of anti-D in ITP patients (Chapter 6). As three out of four of the anemia-causing anti-RBC antibodies were found protective of passively induced ITP in mice, these results add to the evidence that challenges the hypothesis that monoclonal antibodies are not useful in treating immune forms of thrombocytopenia [62-64]. These three antibodies all target public antigens (GPA, band 3 and Kell) expressed by almost all human RBC, suggesting the possibility of an alternative to anti-D in the use of D-negative patients.

In conclusion, results in this thesis show in a murine model of AHA that antibody-mediated mechanisms of anemia may be independent of complement or macrophage classical pathways, and that the beneficial effect of IVIg is primarily displayed in FcγR-dependent anemia. Efficacy
of monoclonal RBC-specific antibodies was demonstrated in the treatment of immune-mediated thrombocytopenia in mice, suggesting that they may provide an effective alternative to anti-D in the treatment of ITP.

### 7.2 Future directions

In future experiments, more antibodies may be studied to investigate the mechanism(s) of anemia. Multiple GPA-specific antibodies will be desired to determine if the agglutination mechanism is a global observation or only specific to a subset of antibodies with certain characteristics (e.g., antibody isotype, specific epitope, etc). Data from the four antibodies assessed in this thesis demonstrated for the first time a potential correlation between FcγR-dependent mechanism of anemia and response to IVIg. Future experiments with more antibodies will be needed to further evaluate this correlation, and most importantly, patient samples (if available) should be tested to see if such a relationship is also valid in the human disease. Finally, this thesis established a platform for the studies of monoclonal erythrocyte-specific antibodies in the amelioration of a mouse model of ITP, and further investigation is warranted for the elucidation of their therapeutic mechanisms of action.
References


74. Crow AR, Suppa SJ, Chen X et al. The neonatal Fc receptor (FcRn) is not required for IVIg or anti-CD44 monoclonal antibody-mediated amelioration of murine immune thrombocytopenia. Blood 2011;118:6403-6406.


Appendices
Appendix 1. **Induction of thrombocytopenia by the anti-GPA antibody.** (A) C57BL/6 mice were injected with the indicated antibody, and bled at the indicated times for platelet enumeration. RBC from untreated mice (●), or mice treated with GPA antibody (45 µg/mouse; □), another erythrocyte-specific antibody anti-CD24 (70 µg/mouse; ◊) or the anti-platelet antibody MWReg30 (2 µg/mouse; ∆) were used to enumerate platelet by flow cytometry. n=3 mice in total from 3 independent experiments. (B) C57BL/6, Fcγ−/−, and C3−/− mice were injected with the GPA antibody, and bled at 24 hours post-injection for platelet enumeration. Platelet counts were expressed as a percentage of initial (baseline) counts. n=3 mice in total from 3 independent experiments.
Appendix 2. Antibody-mediated anemia in surgically splenectomized mice. Normal and surgically splenectomized mice were injected with 70 μg/mouse anti-CD24 (A) or 50 μg/mouse anti-band 3 (B) antibody. Blood from untreated mice (normal: Δ; splenectomized: ▲) or mice treated with antibody (normal: □; splenectomized: ■) were used to enumerate RBC by flow cytometry. n=2 mice in total from 2 independent experiments. The asterisks (*P<.05, **P<.01) refer to statistical significance between normal (□) and splenectomized (■) mice treated with antibody.
Appendix 3. IVIg rescues CD1 mice from anti-GPA antibody-induced death. CD1 mice were injected with 50 μg anti-GPA on day 0. The indicated group of mice also received 2.5g/kg IVIg injected 24 hour prior to the injection of the GPA antibody. n=8 mice in total from 4 independent experiments.
Appendix 4. Sensitization of mouse RBC with the GPA antibody in vivo. C57BL/6 mice were injected with the GPA antibody (45 µg/mouse) on day 0, and bled at the indicated times. RBC from the mice were stained with a fluorescent secondary antibody, and analyzed by flow cytometry for percentage circulating antibody-positive RBC (A) and mean fluorescence intensity (B). n=2 mice in total from 2 independent experiments.
Appendix 5. Sensitization of mouse RBC with the band 3 antibody *in vivo*. C57BL/6 mice were injected with the band 3 antibody (50 µg/mouse) on day 0, and bled at the indicated times. RBC from the mice were stained with a fluorescent secondary antibody, and analyzed by flow cytometry for percentage circulating antibody-positive RBC (A) and mean fluorescence intensity (B). n=2 mice in total from 2 independent experiments.
Appendix 6. Sensitization of mouse RBC with the Kell antibody *in vivo*. C57BL/6 mice were injected with the Kell antibody (50 µg/mouse) on day 0, and bled at the indicated times. RBC from the mice were stained with a fluorescent secondary antibody, and analyzed by flow cytometry for percentage circulating antibody-positive RBC (A) and mean fluorescence intensity (B). n=2 mice in total from 2 independent experiments.
Appendix 7. Peripheral reticulocyte counts increase exponentially in response to treatment with the GPA antibody. C57BL/6 mice were injected with the GPA antibody (TER119; 45 µg/mouse), and bled at the indicated times for absolute reticulocyte counts by supravital staining with new methylene blue. n=1 in total from 1 experiment.
Appendix 8. IVIg does not cause hemolysis in mice. C57BL/6 mice were injected with 2.5g/kg IVIg or albumin intravenously, and RBC counts from untreated mice (Nil; ○), or mice treated with albumin (□) or IVIg (◊) were determined 1 and 2 days post injection. n=4 mice in total from 2 independent experiments.
Appendix 9. The CD24 antibody fails to ameliorate thrombocytopenia induced by MWRReg30 regardless of the manufacturer. C57BL/6 mice were injected with the CD24 antibody (50 µg) from either BD PharMingen (BD) or eBiosciences (eBio). 24 hours later, mice were injected with 2 µg of the anti-platelet antibody MWRReg30 to induce thrombocytopenia. After a further 24 hours, platelet counts were determined on a flow cytometer. n=4 mice in total from 2 independent experiments.
Appendix 10. The anti-CD44 antibody KM114 does not ameliorate passive AHA mediated by the band 3 antibody in mice regardless of treatment regime. C57BL/6 mice were injected with the band 3 antibody at time 0 to induce anemia, and the indicated group also received treatment with the KM114 antibody (BD Biosciences). Each arrow indicates an intravenous infusion of 50 µg of KM114 into mice in the treatment group. Shown are peripheral RBC counts in untreated mice (Nil; ●), and mice treated with band 3 antibody only (□) or Km114 + band 3 antibody (▲). n=2 in total from 1 experiment. (A) One injection of 50 µg KM114 was given 24 hours prior to the injection of the band 3 antibody. (B) One injection of 50 µg KM114 was given 24 hours post anti band 3 injection. (C) 3 injections of KM114 (50 µg each) was given to mice: one at 24 hours prior to band 3 injection, one at 1 day post band 3 antibody injection, and a third one at 3 days post band 3 antibody injection.
Appendix 11. Transfusion of aged or heat-damaged RBC was associated with modest increase in platelet counts following induction of ITP by MWReg30. RBC was harvested from C57BL/6 (A) or BALB/c (B) donor mice by retro-orbital bleeding into CPDA, buffy coat removed, and stored at 75% hematocrit at 4°C for indicated period of time. On the day of RBC transfer, an equivalent of 2 units of aged or heat-damaged RBC was transfused into C57BL/6 recipient mice through tail vein injection. ITP was induced in mice with 2 µg of MWReg30 per mouse, 2 hours post RBC transfusion, and platelet counts were measured 2 hours post
MWReg30 injection. (A) Syngeneic RBC transfer from C57BL/6 donor to C57BL/6 recipient mice. n=4~8 in total from 2~4 independent experiments. B) Allogeneic RBC transfer from BALB/c donor to C57BL/6 recipient mice. n=2 in total from 1 experiment.