Optimal Conditions for the Monocyte Monolayer Assay and Applications of the Assay

by

Tik Nga Tong

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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Tik Nga Tong
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Laboratory Medicine and Pathobiology
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Abstract

Immune-mediated hemolysis, whether due to transfusion of incompatible red blood cells (RBCs) in hemolytic transfusion reactions or passive transfer of anti-RBC antibodies through intravenous immunoglobulin (IVIG) therapy, contributes to complications and comorbidities in patients. A major mechanism of RBC destruction is Fc gamma receptor (FcγR)-dependent phagocytic removal of antibody-bound RBCs by monocyte-macrophage within the mononuclear phagocyte system. The monocyte monolayer assay (MMA) assesses FcγR-mediated destruction using primary monocytes from whole blood and predicts in vivo survival of serologically incompatible, allogeneic RBCs. Optimal conditions for MMA were examined in this thesis. Whole blood collected in acid citrate dextrose anticoagulant and stored at room temperature for up to 36 hours best preserved monocyte phagocytic function. Such conditions were validated using clinical samples from patients receiving IVIG. The findings suggest stored autologous patient samples yield clinically relevant MMA results, and thus permit the shipping and testing of properly stored samples.
Acknowledgments

The three year master's degree has been a roller coaster ride, which constantly forced me to reflect on whether the decision to relocate from beautiful Vancouver and away from my family to Toronto was a good choice. And looking back at the life experiences, growth and handful of genuine friendships I have gained, I do not regret my decision.

The first person I would like to sincerely thank is my supervisor, Dr. Donald Branch. As I was desperately looking for a new laboratory to continue my studies, Don opened his doors for me and accepted me despite the situation I was in. The research part was anything but a smooth sail, but Don was always understanding and encouraging. Other than being a good mentor, Don is quite the fatherly figure, whom I secretly refer to as "Papa Don". I also secretly call Don's office "the black hole", where I often have two-hour meetings, with the first 30 minutes of scientific discussion followed by 90 minutes of good ol' days talk (which I actually enjoy listening to). Lastly, thank you Don for letting me take time off from laboratory work to enrich my overall graduate experience, whether I was taking additional courses for fun, spending 2 months doing research in Taiwan, or travelling.

I would also like to thank my advisory committee, Dr. Alan Lazarus and Dr. Jeannie Callum, for their support and critical suggestions throughout my project.

Next, I would like to thank my parents and my brother, Paul, for always being there for me despite the distance, time zone differences and our own busy schedules. Thank you for always respecting and being supportive of my life decisions even when we do not see eye to eye, and giving me the freedom and confidence to explore the world. And I know you will always be there for me when I am tired from my adventures and needed a break from reality.

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<td>ACD</td>
<td>Acid citrate dextrose</td>
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<tr>
<td>AHG</td>
<td>Anti-human globulin</td>
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<td>AIHA</td>
<td>Autoimmune hemolytic anemia</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>CLT</td>
<td>Chemiluminescence test</td>
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<td>CR</td>
<td>Complement receptors</td>
</tr>
<tr>
<td>DAT</td>
<td>Direct antiglobulin test</td>
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<tr>
<td>DHTR</td>
<td>Delayed hemolytic transfusion reaction</td>
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<td>EXM</td>
<td>Electronic crossmatch</td>
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<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
<tr>
<td>Fc region</td>
<td>Fragment crystallizable region</td>
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<tr>
<td>FMH</td>
<td>Fetal maternal haemorrhage</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HbA</td>
<td>Hemoglobin A (normal)</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HbS</td>
<td>Hemoglobin S (sickle cell variant)</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HDFN</td>
<td>Hemolytic disease of the fetus and newborn</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HSC</td>
<td>Hospital for Sick Children</td>
</tr>
<tr>
<td>HTRs</td>
<td>Hemolytic transfusion reactions</td>
</tr>
<tr>
<td>IAT</td>
<td>Indirect antiglobulin test</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IS XM</td>
<td>Immediate spin down crossmatch</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibition motif</td>
</tr>
<tr>
<td>ITP</td>
<td>Immune thrombocytopenia</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
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<tr>
<td>K$_2$EDTA</td>
<td>Potassium ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MMA</td>
<td>Monocyte monolayer assay</td>
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<td>MPS</td>
<td>Mononuclear phagocyte system</td>
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<td>MSH</td>
<td>Mount Sinai Hospital</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PRBC</td>
<td>Phagocytosed red blood cell (mean phagocytic index)</td>
</tr>
<tr>
<td>R&lt;sub&gt;2&lt;/sub&gt;R&lt;sub&gt;2&lt;/sub&gt; RBC</td>
<td>DcE/DcE phenotyped red blood cell</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute medium-1640</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SB</td>
<td>Sunnybrook Hospital</td>
</tr>
<tr>
<td>SCD</td>
<td>Sickle cell disease</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SHIP1</td>
<td>SH2 domain-containing inositol 5'-phosphatase I</td>
</tr>
<tr>
<td>SMH</td>
<td>St. Michael's Hospital</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen-associated tyrosine kinase</td>
</tr>
<tr>
<td>TRALI</td>
<td>Transfusion related acute lung injury</td>
</tr>
<tr>
<td>UHN</td>
<td>Toronto General Hospital/University Health Network</td>
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<tr>
<td>WR</td>
<td>Weak reaction</td>
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Chapter 1

Introduction

1.1 Overview

Red blood cell (RBC) transfusion remains an important medical procedure used to rapidly increase the level of hemoglobin in a patient to ensure sufficient oxygenation levels in blood. This life saving procedure is well documented in patients with trauma, surgical complications, cardiovascular diseases, hemoglobinopathies, and autoimmune hemolytic anemias. However, RBC transfusion has its associated risks, such as risk for infection, immunological responses, and other adverse events. Despite stringent screening and testing for infectious agents in blood products, newly emerging pathogens and window period diseases are still a concern; although, according to the Canadian Blood Services external surveillance report in 2015, the estimated risks of potentially infectious donations remained very low (e.g. 1 in 21.4 million donations for human immunodeficiency virus [HIV], 1 in 12.6 million for hepatitis C virus [HCV] and 1 in 7.5 million for hepatitis B virus [HBV]) and no transfusion transmitted infections were identified (Surveillance Report, 2015). Other adverse events include febrile reactions, transfusion-associated circulatory overload or dyspnea, transfusion-associated acute lung injury (TRALI) and metabolic disturbances (e.g. hypothermia, hyperkalemia, acidosis) (Harvey et al, 2015; Piccin et al, 2015; Rogers et al, 2016; Clarke, 2011). However, the focus of this thesis will be on hemolytic reactions following transfusion and how to avoid these potential reactions in the face of patients having auto- or alloantibodies to donor RBCs or in the passive transfer of RBC alloantibodies to patients from plasma-derived blood products, such as IVIG.
1.2 Immune-Mediated Hemolysis

Human RBCs have a typical life span of 90-120 days in circulation, and as the primary oxygen carrier, they are continually exposed to oxidative stress that contributes to their aging or senescence (Lutz & Bogdanova, 2013). Senescent RBCs have altered plasma membrane compositions that are recognized and phagocytosed by the tissue macrophages within the mononuclear phagocyte system (MPS) as part of their normal life cycle (Kay & Goodman, 2004; Eckstein et al., 2015). Immune-mediated hemolysis occurs when the immune system targets RBCs for premature clearance by a humoral antibody response, resulting in shortened RBC lifespan and anemia in affected individuals.

1.2.1 Mechanism of Intravascular and Extravascular Hemolysis

Immune-mediated hemolysis can be further categorized into intravascular hemolysis within the blood vessels and extravascular hemolysis within the MPS in the spleen and/or liver (Figure 1.1). This immune-mediated destruction of RBC is caused by two mechanisms: complement activation and/or antibody-mediated phagocytic clearance (Barcellini, 2015; Rosse et al., 2004; Flegel, 2015). The complement system can be activated through three different pathways, all of which converge to the formation of C3 convertase and eventual formation of the C5-9 membrane attack complex (MAC) which results in cytolysis (Chapin et al., 2016; Yazdanbakhsh, 2005). The pathway mainly involved in transfusion medicine is the classical pathway of complement activation cascade, initiated when an antibody-antigen complex, in this case, the antibody-sensitized RBCs, binds the C1q complex (Chapin et al., 2016; Yazdanbakhsh, 2005). This results
in either MAC formation and direct lysis of RBCs within the circulation (intravascular lysis), or the generation of opsonins C3b and C4b, which are able to augment extravascular phagocytosis via binding to complement receptors (CR) (Chapin et al., 2016; Yazdanbakhsh, 2005; Barcellini, 2015). Both IgG and IgM antibodies are capable of activating the complement system via the classical pathway, although IgM is a more potent activator (Atkinson & Frank, 1974; Vidarsson et al., 2014). Downstream C3b opsonins target bound RBC for CR1- and CR3-mediated phagocytosis by tissue macrophages within the MPS (Barcellini, 2015). On the other hand, antibody-mediated extravascular hemolysis usually involves IgG antibodies that target RBCs for Fc gamma receptor (FcγR)-mediated phagocytosis by macrophages within the MPS. Occasionally, IgG antibodies are able to partially activate complement, leading to C3b opsonin generation but not MAC formation (Chapin et al., 2016; Yazdanbakhsh, 2005; Barcellini, 2015; Vidarsson et al., 2014). When both IgG and complement opsonins are present, the phagocytosis can be accelerated and the removal of RBCs occurs primarily in the liver (Petz & Garratty, 2004). Indeed, the mechanisms of RBC destruction mediated by IgG, IgM and complement components are very distinct.
Figure 1.1 Immune mediated intravascular and extravascular hemolysis.

Immune mediated hemolysis is preceded by the binding of IgM and/or IgG molecules on RBC surfaces (sensitization). Intravascular hemolysis can be mediated by the IgM or IgG activation of the complement cascade, leading to formation of the membrane attack complex (MAC) on RBC surfaces and eventual RBC rupture within the blood vessels. On the other hand, extravascular hemolysis occurs within the mononuclear phagocyte system (MPS) located primarily in the spleen and liver, and is mediated by FcyR-dependent phagocytosis of IgG opsonized RBCs.

[Adapted with permission from both Transfusion and the original contributor. Cover of Transfusion Special Issue: Hemolysis Supplement Strategies to Address Hemolytic Complications of Immune Gobulin Infusions (2015). 55(S2); Illustrated by D'Aguilar.]
1.2.2 The Involvement of IgM in Immune-Mediated Hemolysis

IgM is a pentamer that binds ligands with low affinity and high avidity. Naturally occurring high titer anti-A and anti-B IgM isogglutinins are significant contributors to complement-mediated hemolysis due to ABO mismatch in RBC transfusions (Branch, 2015). Other non-ABO anti-RBC IgM antibodies are usually considered benign and clinically insignificant due to optimal reactivity at low temperatures (cold agglutinins), with exceptions in scattered published case studies of hemolysis (Chandeysson et al., 1981; Molthan et al., 1984; Ramos et al., 1994; Arndt et al., 1998; Campbell et al., 2000). In vitro IgM sensitized and chromium-51 radiolabelled autologous RBCs have been shown to be sequestered by the liver post-infusion, while some are destroyed, others are later released back into circulation without detrimental changes in their lifespan (Schreiber & Frank, 1972; Atkinson & Frank, 1974). This has been linked to IgM binding on RBC surfaces below the threshold for complete complement activation, and hence results in C3b-mediated sequestration but no destruction of sensitized RBCs (Atkinson & Frank, 1974). However, with sufficient RBC sensitization as happens with a high antigen site density, such as occurs with anti-A or -B binding to the respective antigen-positive RBCs (Branch, 2015), bound IgMs become potent activators of complement via the classical pathway, resulting in rapid destruction of RBCs intravascularly.
1.2.3 The Role of IgG and FcγR in Phagocytic Clearance of RBC

There are four IgG subclasses in humans, IgG1, 2, 3 and 4, that are named in order of abundance found in the circulation, which is 60%, 32%, 4% and 4%, respectively (Vidarsson et al, 2014). After binding to its cognate antigen, the fragment crystallizable (Fc) region of the four IgG subclasses are recognized and bound by different FcγRs with different affinities (Figure 1.2) (Bruhns, 2012). Monocyte-macrophages, cell populations involved in extravascular hemolysis within the MPS, express four activating FcγRs (e.g. FcγRI, FcγRIIA, FcγRIIC and FcγRIIIA) and one inhibitory FcγRIIB, that bind IgG to mediate downstream immune effector functions of phagocytosis and cell activation (Vidarsson et al, 2014; Bruhns, 2012; Guilliams et al, 2014). Activating FcγRs signal through the immunoreceptor tyrosine-based activation motif (ITAM) results in the activation of Src-family kinases and the spleen-associated tyrosine kinase (Syk) signalling pathway (Vidarsson et al, 2014; Gessner et al, 1998). Inhibitory FcγRIIB signals through an immunoreceptor tyrosine-based inhibition motif (ITIM) and activates downstream signalling through the recruitment of SH2 domain-containing inositol 5'-phosphatase I (SHIP1) which down regulates the activated tyrosine kinases resulting in inhibition of phagocytosis (Vidarsson et al, 2014; Bruhns, 2012; Guilliams et al, 2014). Recently, a new hypothesis suggests that it is the relative strength of activating and inhibitory signals that controls the threshold for phagocyte activation and function (Guilliams et al, 2014). Adding to the complexity, various FcγR polymorphisms have been identified and have altered affinities for IgG subclasses (Bruhns et al, 2009) and certain variants have been associated with disease development and progression (Li et al, 2009).
In relation to immune-mediated hemolysis, IgG2 and IgG4 are rarely implicated despite their clinical significance in other immune related pathologies (Gehlhar et al, 1999; Martinez et al, 2005; Lane & MacLennan, 1986; Carruthers et al, 2012). This is likely due to their poor ability to activate complement and FcγRs (Vidarsson et al, 2014). On the other hand, IgG1 and IgG3 are frequently implicated in hemolysis, due to their abilities to activate complement and FcγRs that are relevant in intravascular and extravascular hemolysis (Pollock & Bowman, 1990; Abramson et al, 1970; Wiener et al, 1987; Vidarsson et al, 2014).
Figure 1.2 Human Fcγ receptors functions and IgG binding affinities.

Schematic representation of human Fcγ receptors at the cell membrane (gray bar) and their associated signalling molecules: immunoreceptor tyrosine-based activation motif (ITAMs; green boxes) or immunoreceptor tyrosine-based inhibition motif (ITIM; white box). The binding affinities of the four human IgG subclasses are indicated: in bold (high affinity), plain (low affinity), between parentheses (very low affinity), or - (no binding).

1.3 Hemolytic Transfusion Reactions (HTRs)

HTRs are mediated by antibodies produced by the recipient's host immune response against transfused RBCs, leading to shortened in vivo survival of the transfused RBCs and reduced transfusion efficacy. In some instances, the severe and rapid destruction of RBCs can be life-threatening, even if mediated by extravascular destruction. HTRs can be further categorized into acute and delayed HTRs. Acute HTRs have a rapid onset within minutes and up to 24 hours following infusion of incompatible RBCs, and are usually mediated by complement proteins and pre-formed anti-RBCs antibodies, resulting in direct lysis of RBCs within the blood vessels (intravascular hemolysis). This usually involves ABO incompatibilities, although antibodies against non-ABO blood group antigens have also been reported to cause acute HTRs (Padmore et al, 2014; Halverson et al, 1994). Delayed HTRs (DHTRs) have onsets between 24 hours to 1 month post transfusion, and they are usually mediated by a secondary immune activation of an evanescent IgG antibody and/or occasionally by a primary IgM antibody response (Public Health Agency of & Canada, 2007; Chandeysson et al, 1981; Molthan et al, 1984). DHTRs are usually mediated by FcγRs or CR-mediated phagocytic removal of antibodies- and/or complement-sensitized RBCs by extravascular hemolysis. In the recent decade, a third type of HTRs called hyperhemolysis has been gaining clinical recognition as a more severe form of DHTR with a different course of clinical manifestations (Petz, 2006). Although the underlying mechanism has yet to be elucidated, hyperhemolysis is characterized by the destruction of both transfused allogeneic and autologous RBCs, that is often coupled with reticulocytopenia, leading to a severe drop in hemoglobin level in patients, below the pre-transfusion level (Darabi & Dzik, 2005; King et al, 1997; Babb et al, 2012; Rogers & Smith, 2014; Yan et al, 2015).
1.3.1 Clinical Markers of Hemolytic Transfusion Reactions

Several clinical symptoms are associated with a post-transfusion hemolytic event, such as pallor, fatigue, shortness of breath, jaundice, and hemoglobinuria (Clarke, 2013). But in order to confirm HTRs, a series of common blood tests are performed. The identification of antibody or complement binding on the RBC surface is an important finding. This can be determined using the direct antiglobulin test (DAT), where secondary anti-IgG and anti-C3d antibodies are added to RBC, and the presence of agglutination would indicate the binding of IgG antibody and/or complement proteins on RBCs in vivo. Furthermore, a class of biomarkers related to blood hemoglobin level and metabolism are examined: a drop in hemoglobin (Hb) level, the oxygen carrying molecule within RBCs, and hematocrit, a measure of the ratio of the volume of RBCs to the total volume of blood; a drop in haptoglobin, a serum molecule that scavenges free hemoglobin to reduce intravascular oxidative damage; and an increase in unconjugated bilirubin, a metabolic breakdown product of hemoglobin that accumulates and overwhelms the body's capacity to conjugate to a soluble form for clearance (Dhaliwal et al., 2004). Another serum biomarker related to cellular or tissue damage is the increase in lactate dehydrogenase (LDH), an intracellular protein that is released upon RBC rupture (Dhaliwal et al., 2004). An increase in reticulocytes, which are immature RBCs produced and released by the bone marrow, is indicative of a negative feedback response to the drop in circulating RBCs and is another marker of hemolysis (Dhaliwal et al., 2004). Additional serological tests are performed to identify the nature of the antibody in immune mediated HTRs that will be discussed in later sections.
1.3.2 Alloimmunization

Aside from acute HTRs that are caused by ABO mismatch, the main mechanism behind HTRs is the phenomenon of alloimmunization. Upon exposure to foreign RBC antigens, due to either allogeneic transfusion, maternal-fetal bleed during pregnancy, and/or transplantation, the recipient immune system mounts an immune response against the foreign antigens on the surface of the RBCs, and thus results in the production of alloantibodies. One consequence of alloimmunization is the increased risk of HTRs and hemolytic disease of the fetus and newborn (HDFN). HDFN happens when a previously alloimmunized mother is carrying a fetus of incompatible blood type, and hemolysis in the fetus occurs when maternal alloantibodies cross the placenta, enter the fetal circulation and destroy fetal RBC through extravascular hemolysis by the fetal/newborn macrophages. Another consequence of alloimmunized patients is the delay in identifying the alloantibodies and suitable antigen-negative RBC units for transfusion that compromises timely patient care and can result in life-threatening situations (Nickel et al, 2016).

1.3.3 Factors that Influence Alloimmunization Rate

There are 23 currently known blood group systems and new blood antigens are still being discovered (Helias et al, 2012; Saison et al, 2012). With the exception of autologous blood transfusion, selected allogeneic donor RBC for transfusion will not be 100% identical to the recipient's RBC genotype. Despite this mismatched blood used in transfusion, only 1-8% alloimmunization rates have been reported for the general population (Chou et al, 2012;
Schonewille et al, 2016). Several factors affect alloimmunization rates: 1) RBC antigen disparity between donor and recipient, e.g., immunogenicity of the RBC antigen, 2) antigen dose, 3) co-existence of inflammatory "danger signals" in recipient, and 4) recipient's immune ability to process and present antigens (Hendrickson et al, 2014).

1.3.3.1 Antigen Disparity between Donor and Recipient, Antigen Dose and Background Inflammation

The factors of RBC antigen disparity, antigen dose and inflammation on alloimmunization rates have been largely examined in patients with sickle cell disease (SCD). SCD is a type of hemoglobinopathy, where a single nucleotide polymorphism of A to T results in the substitution of glutamic acid to valine in the β-globin gene, resulting in the disease variant hemoglobin S (HbS) as opposed to the normal hemoglobin A (HbA) (Rees et al, 2010). The consequence of this single mutation is the aggregation of HbS during the deoxygenation state, leading to the characteristic sickle-shaped RBC (Ballas, 2002). The deformed RBCs aggregate in capillaries and cause painful vaso-occlusive crisis in affected patients. Lifelong transfusion remains the primary treatment for SCD patients and aims to dilute the sickle-shaped RBCs with normal RBCs and thus reduces symptoms.

SCD patients have been known to have higher alloimmunization rates than the general population and therefore represent a special group for investigating alloimmunization rates and mechanisms. First, there are major RBC antigen disparities between SCD patients of mainly African origin, and donors of Caucasian ethnicity (Noizat-Pirenne, 2013). It has been well
acknowledged that there are ethnic differences in RBC antigen prevalence, especially the prevalence of RH polymorphisms within the African population (Brecher et al, 2002; Chou & Westhoff, 2011). Indeed, with extended RBC phenotype matching, there are reduced alloimmunization rates in transfused SCD patients, from 18-76% with ABO and D antigen matching only, to 5-11% with additional C, E and K antigen matching, and down to 0-7% with further extended antigen matching (Chou et al, 2012; Castro et al, 2002). The cumulative data suggest that by closing the RBC antigen differences between donor and recipient, alloimmunization rates are reduced.

The lifelong dependence on transfusion as a primary treatment exposes SCD patients to high cumulative doses of foreign RBC antigens in addition to the inherent ethnic disparity mentioned above. A Dutch study that examined previously non-transfused and non-alloimmunized patients found that the increase in cumulative immunization incidences upon receiving non-extended matched RBC transfusions is associated with increased alloimmunization rates (Evers et al, 2016). In addition, background inflammation inherent to SCD has also been linked to higher alloimmunization rate, where the already primed immune system is more likely to respond to foreign antigens (Damanhouri et al, 2015; Owusu-Ansah et al, 2015; Mendonça et al, 2016).

1.3.3.2 Immune Status of Blood Transfusion Recipient

The last important factor is the immune status of the patient at the time of transfusion. Indeed, the mechanism of alloimmunization depends on the host’s ability to process and present foreign RBC antigen that results in a humoral immune response and alloantibody production. As
aforementioned, a primed immune system due to sterile inflammation in SCD patients or immune activation has been suggested to predispose patients to alloimmunize at a higher rate (Rosse et al, 1990; Talano et al, 2003; Chou & Westhoff, 2011; Noizat-Pirenne, 2013; Körmöczi & Mayr, 2014). Theoretically, immunosuppression by interfering with antigen-presentation and/or preventing antibody production can minimize or even prevent alloimmunization. A few retrospective human case studies using immunomodulatory drugs, such as intravenous IgG (IVIG), corticosteroids, and/or rituximab in conjunction with erythropoietin showed successful management of delayed HTRs and hyperhemolysis in SCD patients (Cullis et al, 1995; Petz et al, 1997; Talano et al, 2003; de Montalembert et al, 2011). However, further systematic investigations are needed to confirm the utility and safety of potential prophylactic immunosuppressants administration in reducing the risks of HTRs.

Other factors that influence alloimmunization have been suggested, such as recipient's genetic predisposition as "responder versus non-responder", donor gender, and RBC storage, but are beyond the scope of this thesis and hence are not reviewed here (Hendrickson et al, 2014; Chassé et al, 2016; Desai et al, 2015; Gehrie & Tormey, 2014).
1.3.4 Clinically Significant versus Insignificant Antibodies

1.3.4.1 Antibody Classification

The clinical consequence of alloimmunization depends on the nature and titer of the alloantibody. An old classification divided alloantibodies into three groups based on their reported clinical significance in causing HTRs and/or HDFN. Group I is always clinically significant and includes antibodies against the Rh, MNS, Kidd, Duffy and Kell blood systems (Schanfield et al., 1981). Group II is considered clinically insignificant due to the lack of reported cases of HTRs and/or HDFN, and it includes anti-Knops (Kn<sup>a</sup>), -York (Yk<sup>a</sup>) and -Chido(Ch<sup>a</sup>) (Schanfield et al., 1981). And Group III contains antibodies of mixed nature (e.g. anti-Cartwright, Yt<sup>a</sup>), where they are sometimes associated with clinical cases of HTRs and/or HDFN, but are sometimes completely benign (Schanfield et al., 1981). As opposed to Group I alloantibodies that should always be considered during transfusion, the classification of Group II alloantibodies might be a misnomer and cause oversights when dealing with these alloantibodies. In fact, there are new published case studies of HTRs caused by alloantibodies that were once believed to be clinically insignificant and thus overlooked during donor RBC crossmatch (O’Reilly et al., 1985; Kumawat et al., 2015; Kaur et al., 2012). One key point is that the presence of alloantibodies does not always result in clinical manifestations and that alloantibodies should be evaluated on a patient-by-patient basis.
1.3.4.2 Factors that Influence Clinical Significance versus Insignificance

The variations in clinical significance of alloantibodies and disease severity can be attributed to: 1) the immunogenicity of the antigen, 2) thermal range, and 3) antibody subclass and its ability to engage the Fc receptor on effector immune cells (e.g., monocyte/macrophages). The immunogenicity of an RBC antigen is related to its ability to be recognized and processed by antigen-presenting cells (APCs), and small peptides of the processed antigen presented in the context of the major histocompatibility II (MHC II); thus, triggering a humoral immune response that is crucial for alloimmunization. From retrospective analyses of published reports of alloantibodies, K, E and Cw are among the most immunogenic antigens after the mandatory matching of ABO and RhD blood groups (Evers et al., 2016; Tormey & Stack, 2009). While the antigen immunogenicity affects alloimmunization rates, the thermal range of alloantibody determine its clinical relevance. Each antibody has its optimal temperature for binding its cognate antigen. The thermal ranges of an antibody is examined by sensitizing antigen-positive RBC with alloantibodies at 37°C ("warm") or 4°C ("cold"). Typically, cold alloantibodies with optimal binding temperature below 25°C are considered non-clinically significant, since they are unable to sensitize RBC and hence unlikely to mediate hemolysis within the normal body temperature range of 28-31°C (Milkins et al., 2013). Lastly, certain alloantibody IgG subclasses, especially IgG1 and IgG3, have been implicated in disease severity, due to their abilities to mediate effector responses relevant in both intravascular and/or extravascular hemolysis (Pollock & Bowman, 1990; Abramson et al., 1970; Wiener et al., 1987). Whether the presence of alloantibodies of a single or a mixture of IgG subclasses predicts hemolysis severity is still
controversial due to seemingly contradicting observations in the literature (Pollock & Bowman, 1990; Sokol et al, 1990).
1.4 Transfusion Compatibility Tests

As exemplified in the previous section, alloimmunization increases the associated risk of HTRs and HDFN. Therefore, it is undisputable that primary alloimmunization should be mitigated by addressing the associated factors. This leads to the implementation of pre-transfusion compatibility tests that detect the presence of auto/alloantibodies and predict transfusion outcome. The aim is to identify the specificity of such antibodies, and to match antigen-negative donor RBCs by serological testing with the implicated antibody, in attempt to reduce the risk of transfusion related complications. Pre-transfusion serological compatibility test results are thus used to identify potentially clinically significant RBC antibodies and to match appropriate donor RBCs for transfusion.

1.4.1 Serology Investigations

According to the Canadian Blood Services guidelines, standard pre-transfusion testing procedures include ABO and Rh typing of patient's RBC, direct antiglobulin test (DAT) (optional), antibody screen of patient's plasma for pre-formed anti-RBC antibodies, and a follow-up antibody identification if the antibody screen is positive (Clarke, 2013).
1.4.1.1 ABO and Rh Blood Group Typing

ABO and Rh are the two most immunogenic blood systems. An ABO mismatch can lead to acute intravascular hemolysis due to the presence of naturally occurring anti-A and anti-B IgM antibodies (Branch, 2015; Roback, 2014). The Rh group contains a complex family of antigens, with the five major antigens as D, C, c, E and e, and the D antigen is the most immunogenic (Roback, 2014). ABO typing is performed via forward typing and confirmed by reverse typing. Forward typing for ABO detects the presence of the A and/or B antigens on patient RBC by adding anti-A and/or anti-B antibodies, and evaluating for the presence of agglutination (Judd et al., 2008; Roback, 2014). Reverse typing involves adding patient serum/plasma, which contains anti-A or -B antibodies, to reference type A (expressing A1 or A2 antigen) and type B cells (Judd et al., 2008; Roback, 2014). For example, a patient with type A blood should have agglutinated RBC upon the addition of anti-A in the forward typing due to the presence of the A antigen, and agglutinated reference type B cells in the reverse typing due to the presence of anti-B antibodies. Rh typing is just using a forward type with anti-Rh(D) antisera.

1.4.1.2 Direct Antiglobulin Test (DAT)

DAT is performed to examine the potential presence of autoantibodies as a result of an autoimmune hemolytic anemia (AIHA), where the autoantibody is sensitizing autologous RBCs, or alloantibodies that are reacting with allogeneic transfused RBCs. The addition of a secondary anti-human IgG antibody to the RBCs would cause agglutination only if autologous RBCs have been sensitized by autoantibodies or if alloantibodies are reacting with recently transfused RBCs.
in vivo. This is often conducted using a tube technique, where naive patient blood is first diluted in buffered saline, then secondary anti-human IgG antibodies are added, and the mixture is centrifuged to pellet the RBCs. A score between 0 to 4+, as a semi-quantification of the level of antibody sensitization, is given based on the agglutinated pellet size(s) that is resistant to resuspension (Judd et al., 2008; Roback, 2014).

1.4.1.3 Antibody Screen

An antibody screen is conducted using patient's plasma or serum against 2 to 3 group O reference RBCs, each phenotyped for the main RBC blood group antigens and expressing most of the clinically significant antigens frequently associated with HTRs. This was once conducted exclusively using the "tube technique", but the gel and solid phase are often employed instead (Judd et al., 2008). The underlying mechanism is that the presence of a specific antibody would lead to agglutination of the corresponding reference RBC expressing the cognate antigen, resulting in a positive readout. For example, the presence of anti-K antibody would result in the agglutination of the K-expressing reference RBCs, but not the K-negative RBCs. Any positive antibody screen will be followed-up by an antibody investigation to identify the antigen specificity using a phenotyped panel of 15-20 group O reference RBCs.
1.4.1.4 The Advantages and Disadvantages of Serology

Two major caveats are associated with serological tests, one is the sensitivity of an agglutination-based test and another is the relevant coverage of the antibody screen. Previous studies have correlated the amount of RBC bound IgG to agglutination test score and found the detection limit to be approximately 100-200 IgG molecules per RBC (Merry et al, 1984; Fabijańska-Mitek et al, 1997). Therefore a negative test result does not necessarily imply the complete absence of IgG binding and its potential clinical significance in HTRs cannot be negated (Gardner et al, 2015; Bloch et al, 2015). Moreover, the routine antibody screen and identification panel only involves the commonly observed clinically significant antigens, where unusual or rare antibodies might be missed (Canadian Blood Services Diagnostic Services Ontario Year in Review, 2015).

1.4.2 Serological Crossmatching

Serological results are used to aid in the selection of antigen-negative donor RBC units for potential transfusion in a process called crossmatching. Three main types of crossmatch (XM) are utilized and all of them evaluate serological compatibility based on the absence of agglutination between recipient's serum and potential donor RBC unit. An immediate spin down crossmatch (IS XM) involves mixing of patient's serum with potential donor RBC units and immediately spinning down the mixture to assess agglutination, intended to confirm ABO compatibility. The 37°C crossmatch involves incubating patient's serum with potential donor RBC units at 37°C before centrifugation and assessing agglutination, and aims to detect the
presence of warm-reacting agglutinating antibodies. If patient serum instead of plasma is used, RBC hemolysins can be directly detected, where alloantibodies directly lyse RBCs due to complement activation. An anti-human globulin crossmatch (AHG XM, also termed the indirect antiglobulin test; IAT) involves the addition of a secondary anti-human globulin antibody following the 37°C crossmatch to amplify any opsonization by primary antibody from patient's serum.

A major problem with the serologic crossmatch is that when incompatibilities are found, the strength of the reaction is not always predictive of an incompatible transfusion. In other words, a 4+ reaction may not result in shortened RBC survival while a weak serologic reaction could create significant *in vivo* hemolysis upon transfusion (Noumsi *et al*, 2015; Branch *et al*, 1984). Thus, investigators have been warned against using a so-called “least incompatible” serologic crossmatch for selection of blood for transfusion in difficult cases (Petz, 2003).

A fourth type of crossmatch is coined electronic crossmatch (EXM), which is only applicable to patients with a negative DAT and a negative antibody screen, where potential donor RBC units are matched using an online inventory based on ABO, Rh and other typing results (Mazepa *et al*, 2014). However, controversies have been raised in regards to EXM due to the sole reliance on clerical accuracy of the electronic database (Arslan, 2006; Padmore *et al*, 2014; Mazepa *et al*, 2014).
1.4.3 Other Laboratory Tests that Assess Transfusion Compatibilities

Other functional techniques have been developed to assess the degree of RBC opsonization and to predict the \textit{in vivo} survival of transfused RBCs; and they are reviewed below.

1.4.3.1 The Chromium-51 (\textsuperscript{51}Cr) Release Assay

One of the earliest functional diagnostic tests for predicting \textit{in vivo} survival of transfused RBCs is the chromium-51 (\textsuperscript{51}Cr) release assay. Chromium is found in abundance within RBC, and previous studies have found that \textsuperscript{51}Cr-labelled sodium chromate (Na\textsubscript{2}\textsuperscript{51}CrO\textsubscript{4}) is readily absorbed and stably retained by RBCs (Gray & Sterling, 1950; Ebaugh \textit{et al}, 1953; Mollison & Veall, 1955). \textsuperscript{51}Cr has a half-life of 27.7 days and the majority (91\%) decays by electron capture, while a small percentage (9\%) of the radionuclide undergo secondary decay that produces gamma emission (Kassis \textit{et al}, 1985). The procedure involves labelling a small amount of donor RBCs with radioactive sodium chromate (Na\textsubscript{2}\textsuperscript{51}CrO\textsubscript{4}), washing and then transfusing the labelled RBC into the potential transfusion recipient. Post-transfusion survival of labelled RBC is monitored by interval sampling of blood and tracking the level of radiation using a scintillation counter. By comparing to the normal clearance rate of healthy RBC, the compatibility of the donor RBC can be assessed based on post-transfusion clearance rate. This assay is very sensitive and has great predictive power because it assesses the \textit{in vivo} survival of a small sample of transfused donor RBCs in recipient. However, due to the use of a radioisotope and lengthy experimental procedure, the \textsuperscript{51}Cr release assay is no longer employed for predicting HTRs.
1.4.3.2 The Rosette Test

The rosette test was originally developed to screen and semi-quantify post-partum fetal maternal hemorrhage (FMH) (Sebring & Polesky, 1982). FMH contributes to the alloimmunization of Rh-negative mothers when carrying a Rh positive fetus. Such alloimmunization leads to the production of anti-Rh(D) antibodies that heightens the risk of hemolytic disease of the fetus and newborn (HDFN) in subsequent pregnancies, where the maternal anti-Rh(D) antibodies cross the placenta to enter fetal circulation, sensitize fetal Rh positive RBC and cause hemolysis in the fetus. Maternal Rh alloimmunization can be prevented by the prophylactic administration of anti-Rh(D) antibodies (RhIgG), wherein the RhIgG bind to any fetal Rh positive RBC and target them for benign clearance by the MPS before the maternal immune system can mount an immune response against the foreign Rh(D) antigen (Hummel, 1972; Woodrow et al, 1975). Hence the rosette test detects the presence of Rh positive RBC with the use of commercially available anti-Rh(D) antibodies, and thus assists the appropriate dosing of RhIgG (Sandler & Gottschall, 2012). The rosette test has been modified to purify FcR-expressing cells and investigate inhibition of ligand-FcR interaction, so the rosette test can theoretically be modified to detect the presence of other alloantibodies besides anti-D (Krammer et al, 1976; Masucci, 1982; Masucci et al, 1983; Hadley et al, 1991). Indeed, the rosette test has been shown to have enhanced sensitivity in detecting sub-agglutinating levels of anti-D, -K and -Le\(^a\) in patient sera than the regular DAT and IAT (Galili et al, 1981). However, the clinical utility of the rosette test remains limited to assessing FMH. This may be due to the criticism that antibodies that cause RBC adhesion do not necessarily cause RBC destruction (Hunt et al, 1981).
1.4.3.3 The Chemiluminescence Test (CLT)

The chemiluminescence test (CLT) was developed as an objective and semi-quantitative method of measurement by exploiting the generation of reactive oxygen species (ROS) during erythrophagocytosis. Oxidative burst by phagocytes is a well-documented phenomenon where upon the trigger of phagocytosis, the normally compartmentalized subunits assemble into the active nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex at the plasma membrane (Forman & Torres, 2002; Dupre-Crochet et al, 2013). Using NADPH as an electron donor, the oxidase reduces oxygen molecules into highly reactive superoxide anions ($O_2^-$). The superoxides then dismutate to hydrogen peroxide ($H_2O_2$) spontaneously or enzymatically by superoxide dismutase (SOD), leading to a transient generation of ROS. However, the short half-life and weak luminescent signal of the generated ROS rendered them difficult for direct detection (Strachan et al, 1996), therefore optical enhancements were utilized during the development of CLT back in the late 1980's and early 1990's. Luminol is a membrane permeant probe that luminesces when it reacts with superoxide anion (Dupre-Crochet et al, 2013). In the two pioneering publications, peripheral blood mononuclear cells (PBMCs), anti-D sensitized RBC (or non-sensitized RBC negative control) and luminol were mixed inside a cuvette and monitored inside a luminometer over time (Hadley et al, 1988; Downing et al, 1990). It was shown that phagocytosis occurred when adherent monocytes and sensitized RhD+ RBCs were mixed, as inferred with a peak luminescence between 35-45 minutes and reaction exhaustion by 120 minutes (Downing et al, 1990). Readout was converted to an opsonic index by dividing the area under the luminescence curve of sensitized RBC by the area of non-sensitized RBC (Hadley et al, 1988; Downing et al, 1990). Indeed, the CLT has been utilized in predicting HDFN severity due to RhD alloimmunization (Lucas et al, 1993; Hadley et al, 1991b; Strachan et al,
1996; Hadley et al, 1998) and the outcome of transfusing incompatible blood (Hadley et al, 1999) with superior clinical correlations when compared to standard serological methods. However, CLT is not free of caveats. In addition to signal interference from the presence of free hemoglobin and phenol red, several practical considerations need to be addressed. First, interval monitoring within a luminometer makes maintenance of physiological temperature and carbon dioxide (CO₂) difficult. Second, there is a discrepancy in the optimal pH for monocyte function (pH 7.4) and luminol-generated chemiluminescence (pH 12.0), leading to the compromised assay pH at 8.0 (Downing et al, 1990).

1.4.3.4 The Monocyte Monolayer Assay

The monocyte monolayer assay (MMA) is the first in vitro functional assay that assesses the effect of anti-RBC antibodies in mediating FcγR-dependent phagocytosis to predict transfusion outcome. Developed back in the early 1980's, the MMA utilizes primary monocyte-macrophage as effector phagocytes to assess the antibody-dependent potential for in vivo RBC destruction of alloantibody bound donor RBC (Hunt et al, 1980; Schanfield et al, 1981; Branch et al, 1984). This can be achieved by isolating PBMCs from whole blood and allowing the adherent monocyte population to form a monolayer in the assay chamber, with a purity of over 95% with few contaminating lymphocytes or neutrophils (Sagone et al, 1976). Then, ideally, patient serum, containing relevant alloantibodies and complement proteins, is used to sensitize potential donor RBCs. The sensitized donor RBCs are co-incubated with the monocyte monolayer to mimic Fc-mediated phagocytosis during extravascular hemolysis. The assay readout of phagocytosis requires manual quantification, which has been critiqued for its subjectivity (Downing et al,
Regardless of the potential caveats, the MMA is still currently an approved reference test offered by the American Red Cross (A Guide to American Red Cross Reference Laboratory Services, 2016). Moreover, the MMA has been used successfully as a crossmatch technique instead of serology to select the “most compatible” blood (based on lowest phagocytosis in vitro) and no HTRs were observed in subsequent transfusions (Noumsi et al, 2015).

1.4.4 Sensitivity and Predictability of Pre-Transfusion Compatibility Tests

The five assays reviewed above have their associated advantages and disadvantages. Serological tests based on agglutination to assess the presence of anti-RBC antibodies have a long history of utility since their first description back in the 1940’s (Coombs et al, 1945). They are still currently employed in reference laboratories due to relative ease of operation and rapid result turnover time. In addition to the caveat of limited sensitivity, the presence of anti-RBC antibodies or strength of serologic activity does not always correlate to clinical outcomes of hemolysis (Branch et al, 1984; Noumsi et al, 2015). Therefore, functional assays, such as the $^{51}$Cr release test, rosette test, CLT and MMA were developed to more accurately assess the clinical significance of anti-RBC antibodies. Although highly sensitive and predictive, the $^{51}$Cr release test is no longer used in clinic due to the requirement of radioisotope and lengthy procedure time. The rosette test, MMA and CLT predict clinical outcomes by assessing the binding of sensitized RBC to Fc receptors on monocyte-macrophages, with readout of rosette formation (adhesion) and/or phagocytosis. Comparison studies of the three assays showed that the assessment of erythrophagocytosis by both MMA and CLT correlated to clinical outcomes more than the rosette test (Hunt et al, 1981; Lucas et al, 1993). While the rosette test is limited to
assessing FMH in reference laboratories, the MMA and CLT are employed in difficult clinical cases when serology alone is insufficient to confidently select the RBC unit for transfusion that has the best chance for in vivo survival.

### 1.5 Other Relevant Considerations in Managing Difficult to Transfuse Patients

Selecting compatible RBC units for transfusion is affected by three components: 1) antigen specificity and nature of the anti-RBC alloantibody, 2) availability of antigen-negative blood, and 3) patient's immune status at the time of transfusion. The selection process is complicated when dealing with difficult to transfuse patients, namely patients with an alloantibody against a high prevalence antigen and/or with multiple alloantibodies (Brzica et al., 1977). High prevalence antigen is defined as an antigen that is expressed in the majority (>90%) of the population, but are often over 99% frequency, and thus render the majority of the population as incompatible donors (Moulds, 2009; Brecher et al., 2002). On the other hand, patients with multiple alloantibodies require extensive RBC donor testing and crossmatching to identify compatible RBC units lacking all the antigens to maximize the success of the in vivo outcome. In both cases, the identification of antigen-negative RBC units is challenging and time consuming. Often, the antigen-negative units cannot be identified, are difficult to obtain or there is only a limited supply.
In addition, the immune status of the patient influences transfusion outcome, whether it is the ability to present antigen and mount a humoral response that risk future alloimmunization, or the ability of effector immune cells to destroy transfused RBC in HTRs. Indeed, patients who are immunosuppressed due to the nature of their primary pathology and/or medication have reduced risk for alloimmunization and HTRs (Zalpuri et al, 2014; Gardner et al, 2015).

1.6 Use of MMA in Other Clinical Application

Another clinical application of MMA is investigating the mechanism of IVIG-associated hemolysis. IVIG is a blood product that is pooled and purified from thousands of donors’ plasma, consisting of mainly IgG, and traces of IgM and IgA (Shehata et al, 2010; Dhainaut et al, 2013). IVIG was originally used to treat primary immunodeficiency, such as hypogammaglobulinemia, by conferring passive immunity through IgG supplementation (Eibl, 2008; Schwartz, 1990; Chipps & Skinner, 1994; Jolles et al, 2005). Immunomodulatory effects of IVIG was discovered after the successful treatment of childhood immune thrombocytopenia (ITP) with high dose IVIG, resulting in the expanded use to successfully treat a diverse array of inflammatory diseases (Imbach et al, 1981; Guidelines and Recommendations for IVIG).

In addition to its clinical efficacy, IVIG use is associated with rare occasions of severe immune-mediated hemolytic anemia (Berard et al, 2012; Berg et al, 2015; Padmore, 2015; Michelis et al, 2014), which has been gaining clinical recognition and attention in recent decades as the demand
for IVIG therapy increases. The precise mechanism of hemolysis remains elusive; however, it has been hypothesized that the presence of anti-A and anti-B isoglutinins within IVIG are capable of sensitizing non-group O RBCs and mediating RBC destruction through complement activation or erythrophagocytosis (Padmore, 2012, 2015; Pendergrast et al, 2015; Bellac et al, 2015). Indeed, the two well recognized risk factors for IVIG-associated hemolysis are non-group O recipients receiving a high dose of IVIG (≥2 g/kg), which support the notion of passive anti-A and anti-B antibodies in RBC sensitization and hemolysis (Kahwaji et al, 2009; Padmore, 2012, 2015; Pendergrast et al, 2015; Welsh & Bai, 2015; Berg et al, 2015). Two retrospective studies revealed the prevalence of IVIG-associated hemolysis in relation to patient blood type: A (72-73%), AB (17-18%), B (7-10%), and O (0-3%) (Berg et al, 2015; Padmore, 2015). The disproportional high hemolysis rates of 72-73% in type A recipients despite a population distribution of only 26-40% offers strong evidence of ABO bias in IVIG-related hemolysis (Berg et al, 2015). However, this simple mechanism does not fully explain the complex etiology, since not all non-group O patients will hemolyze despite RBC sensitization, and rare group O patients can hemolyze despite the lack of A and B antigens (Welsh & Bai, 2015; Michelis et al, 2014). Therefore, additional contributing factors must be involved (Padmore, 2012; Pendergrast et al, 2015). Indeed, this is supported by a published case study by Michelis et al, where the MMA was used to examine the mechanism of severe hemolysis in an ABO mismatched bone marrow transplant patient receiving IVIG for her immune thrombocytopenia (ITP) (2014). The MMA results revealed that the use of autologous patient cells was associated with heightened RBC destruction by mononuclear phagocytes, but not when allogeneic cells were used, which was suggestive of pre-activation of the MPS resulting in the observed severe hemolysis in vivo (Michelis et al, 2014).
1.7 Rationale and Hypothesis

Conventional approaches to RBC transfusion are still largely based on serological compatibility and the identification of antigen-negative RBC unit. This approach is straightforward and pertinent to a majority of the transfusion cases with no clinical consequences of alloimmunization and/or HTRs. However, the serological approach is complex and time-consuming when dealing with a small group of patients that are classified as "difficult to transfuse" due to the presence of an alloantibody against a high prevalence antigen and/or the presence of multiple auto/alloantibodies. To further complicate the situation is that not all alloantibodies are clinically relevant and able to cause HTRs. Therefore, the clinical manifestation extends beyond the simple presence of anti-RBC antibodies provided by serological tests. The correlation between serology and clinical hemolysis is far from perfect, with publications highlighting the discrepancy in numerous cases: a strong antiglobulin test with no hemolysis and a negative antiglobulin test with severe hemolysis (Branch et al., 1984; Bedi et al., 2014; Meulenbroek et al., 2015). Even a negative antiglobulin test does not imply the complete absence of a clinically significant antibody binding (Bloch et al., 2015). The observation that a strong antiglobulin test in conjunction with the absence of hemolysis supports the notion that not all anti-RBC antibodies are clinically significant (Branch et al., 1984; Noumsi et al., 2015). Therefore, there is a need to assess the ability of anti-RBC antibodies to mediate downstream effector functions, namely Fc receptor-mediated phagocytosis.

Of the available functional assays, the MMA was chosen as the focus of this thesis. The $^{51}$Cr release assay and the rosette test were not considered due to issues with research ethics and limited sensitivity, respectively. The decision between CLT and MMA was based on the
evaluation of assay conditions and future clinical utility. With similar assay sensitivity, the CLT has an optimal assay pH at 8.0, while the MMA can be performed at physiological pH of 7.2, where the maintenance of physiological conditions is likely crucial for accurate assessment of phagocytosis. In addition, the clinical utility of MMA in predicting transfusion outcomes was highlighted by successful RBC transfusions that were based solely on a pre-transfusion MMA compatibility test, or the MMA crossmatch (Noumsi et al, 2015). In contrast, previous publications of CLT are based on retrospective investigation of hemolysis case studies (Hadley et al, 1999).

With the factors of patient immune status and Fc receptor polymorphisms, several groups have advised the use of autologous patient PBMC in the MMA to more accurately predict transfusion outcomes (Hunt et al, 1982; Branch et al, 1983; Gallagher et al, 1983; Pérez et al, 1986; Branch, D.R., Gallahger, 1986; Napier & Rowe, 1987; Mendoza et al, 1991; Zupańska et al, 1995; Michelis et al, 2014). However, the convention of using fresh samples poses logistical problems of proper sample handling and time of delivery from the blood clinic to the reference laboratories. Whether the processing of blood samples can be delayed while maintaining the validity and predictive power of the MMA has not been investigated.
Herein, I hypothesize that conditions can be found whereby blood samples can be stored while maintaining monocyte function in the MMA for both clinical and research purposes. There are two aims to test my hypothesis:

**Aim 1:** Optimize the whole blood collection and storage conditions that best preserves monocyte phagocytic function for the purpose of MMA using healthy donor blood.

**Aim 2:** Assess the optimized conditions in clinical applications of the MMA using patient cases.
Chapter 2

Optimal Conditions for the Performance of a Monocyte Monolayer Assay

Tik Nga Tong,1,2 Emerald Burke-Murphy,2 Darinka Sakac,2 Jacob Pendergrast,3 Christine Cserti-Gazdewich,3 Vincent Laroche4 and Donald R. Branch1,2,3,5

From the 1Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada; 2Centre for Innovation, Canadian Blood Services, Toronto, Ontario, Canada; 3University Health Network, Toronto, Ontario, Canada; 4Centre Hospitalier Affilié Universitaire de Québec, Québec City, Québec, Canada; and 5Department of Medicine, University of Toronto, Toronto, Ontario, Canada

The work presented in this chapter has been published in Transfusion (2016). Permission for republication has been obtained. The manuscript has been reformatted and modified for better uniformity within this thesis. TN Tong performed the majority of the experiments, E Burke-Murphy performed one donor replicate for Figure 2.3, and D Sakac conducted experiment for Figure 2.5E. J Pendergrast, C Cserti-Gazdewich and V Laroche provided the clinical samples for Figure 2.5. TN Tong wrote the manuscript, and both TN Tong and DR Branch were involved in the revision process.
2.1 Abstract

Background: Various versions of the MMA have been used to assess clinical significance of RBC alloantibodies in transfusion for more than 35 years. However, the optimal conditions, including anticoagulant used for whole blood samples, temperature and duration of storage, and optimal pH for assessing the response of monocytes to antibody-bound RBCs, have never been clearly delineated. Study design and methods: Whole blood from healthy donors was collected in ACD, EDTA, or heparin and stored at room temperature (RT) versus 4°C for up to 2 days. pH was examined with and without buffers. Phagocytosis of anti-D–opsonized R₂R₂ RBCs was used as the positive control for comparison studies. Whole blood was taken into ACD and kept at RT until testing, from patients with or without immune hemolytic anemia. Results: No significant differences in the phagocytosis of the R₂R₂ control RBCs were observed using ACD anticoagulant between freshly drawn or up to 36-hour stored whole blood kept at RT, regardless of the donor. Physiologic pH during MMA was important for optimal monocyte interactions with antibody-opsonized RBCs. MMA results with patient samples, under optimal conditions, kept up to 30 hours in one instance of long-distance shipment, correlated with clinical hemolysis. Conclusion: MMA can be reliably performed on whole blood samples drawn into ACD and kept at RT for up to 36 hours and when physiologic pH is maintained during the assay. Future studies are required to confirm whether use of these conditions with patient monocytes can provide accurate determination of alloantibody significance in patients requiring blood transfusion.
2.2 Introduction

The MMA has been used for more than 35 years to predict the hemolytic significance of a RBC auto- or alloantibody (Schanfield et al, 1980; Hunt et al, 1980; Schanfield et al, 1981; Hunt et al, 1981; Conley et al, 1982; Hunt et al, 1982; Gallagher et al, 1983; Branch et al, 1983; Alperin et al, 1983; Branch et al, 1984; Pérez et al, 1986; Branch, D.R., Gallahger, 1986; Zupanska et al, 1987; Nance et al, 1987; Mendoza et al, 1991; Lucas et al, 1993; Zupańska et al, 1995; Arndt & Garratty, 2004; Noumsi et al, 2015; Michelis et al, 2014; Napier & Rowe, 1987; Fabron et al, 2004; Garratty, 1990). In recent years, this assay has been adapted to examine IVIG implicated in post administration hemolysis (Michelis et al, 2014). However, despite the long history of the assay's use, versatility, and predictive powers, the optimal conditions for storage of whole blood and/or PBMCs before the performance of the MMA have never been adequately addressed. Use of autologous patient (rather than allogeneic donor) blood has been advised so as to more accurately reflect individual differences in immune status and Fc-receptor polymorphisms, and thereby improve the predictive power of the assay (Hunt et al, 1982; Gallagher et al, 1983; Branch et al, 1983; Alperin et al, 1983; Branch et al, 1984; Pérez et al, 1986; Branch, D.R., Gallahger, 1986; Zupańska et al, 1995; Michelis et al, 2014; Napier & Rowe, 1987). However, most investigators do not use autologous monocytes, mostly due to a belief that the MMA has to be done using freshly drawn blood, and so use allogeneic monocytes (Nance et al, 1987; Garratty, 1990; Arndt & Garratty, 2004; Noumsi et al, 2015). It has been claimed that whole blood drawn into heparin anticoagulant and kept at RT storage for up to 36 hours does not compromise monocyte function and allows for autologous monocytes to be examined, even transported over long distances; however, this remains controversial (Branch et al, 1984; Alperin
et al, 1983; Garratty, 1990). Although the data to support the use of autologous patient whole blood for the MMA are relatively strong, there are no published data to adequately address this issue, nor optimal anticoagulant or length of storage to use in the MMA (Hunt et al, 1982; Gallagher et al, 1983; Branch et al, 1983; Alperin et al, 1983; Branch et al, 1984; Pérez et al, 1986; Branch, D.R., Gallahger, 1986; Mendoza et al, 1991; Zupańska et al, 1995; Michelis et al, 2014; Napier & Rowe, 1987).

We sought to systematically investigate the optimal anticoagulant and storage conditions for whole blood, as well as the usefulness of isolating PBMCs before the MMA, and the role of maintenance of physiologic pH during the MMA, to determine how best to maintain the optimal predictive value of antibody-opsonized RBC interactions with monocytes. The identified optimal conditions were further validated in patient case studies. The limits and constraints for pre-MMA specimen management and assay conditions may thus inform quality practices and procedures for this test.
2.3 Materials and Methods

2.3.1 Healthy Donor Blood Samples

Eleven healthy donors, five males and six females, between the ages of 20 and 60 years were recruited for this study. After informed consent was obtained (Canadian Blood Services Research Ethics Board [REB] approvals 2005.002 and 2005.003), donor whole blood was collected via venipuncture using Vacutainer tubes containing acid citrate dextrose (ACD), ethylenediaminetetraacetic acid (K₂EDTA), or lithium heparin (BD). Samples were collected in ACD as a default best anticoagulant unless otherwise indicated (Hess, 2006).

2.3.2 Patient Blood Samples

Patients receiving 2 g/kg IVIG therapy for their underlying conditions were recruited to participate in a study investigating post IVIG infusion idiosyncratic hemolysis. Four patients, two having clinical evidence of IVIG-associated hemolysis and two non-hemolyzers, were from University Health Network (local; REB 14-8191-BE) and one patient with clinical evidence of IVIG-associated hemolysis was from Quebec City (REB approval 2012-1553, PEJ-616), more than 800 kilometers away from site of testing. Pre- and post-IVIG infusion patient RBCs were collected into EDTA tubes and stored at 4°C. A follow-up blood sample 5 to 10 days after IVIG
infusion, or when patients presented with hemolysis, was collected into ACD tubes for PBMC isolation. All ACD samples were stored at RT for up to 30 hours before testing due to logistic or geographical reasons. As previously described by Michelis et al (2014), patient RBCs collected before and after IVIG infusion were opsonized with an \textit{in vitro} 2 g/kg dosage of IVIG (a laboratory lot was used for the UHN patients, and the actual lot of IVIG infused was used for the Quebec City patient, according to the conversions outlined in table A2). Autologous PBMCs were used in patient MMA, and where possible, a storage-matched allogeneic MMA using monocytes from healthy controls was performed in parallel. Anti-D–opsonized R\textsubscript{2}R\textsubscript{2} RBCs were used as a positive phagocytosis control (see below). Cutoff, above which gives a significant reaction with IVIG, has been previously described as a phagocytic index of more than 17 (Michelis \textit{et al}, 2014).

\textbf{2.3.3 Whole Blood Storage at RT and 4°C}

ACD anticoagulant was initially chosen for these studies due to its previously reported benefits for preservation of whole blood (Hess, 2006). Whole blood from the same donor was drawn together into multiple ACD tubes (yellow top) and either designated for processing immediately (fresh isolation, $T = 0$ hr) or stored at RT and/or 4°C for 24, 36, and 48 hours before the isolation of PBMCs) by Ficoll-Paque as recommended by the manufacturer (GE Healthcare). RT is defined as ambient temperature, which was between 18 and 22°C during this study.
2.3.4 Whole Blood Storage in Different Anticoagulants

To examine the effects of different anticoagulants in each donor, whole blood was collected into ACD, EDTA (lavender top), and heparin (green top) tubes. Whole blood was either processed immediately (fresh isolation, T = 0 hr) or stored at RT for 24, 36, and 48 hours on a stationary rack before PBMC isolation.

2.3.5 MMA

MMA was performed as previously described with minor modifications (Purohit et al, 2014; Rampersad et al, 2005). Briefly, PBMCs were isolated from whole blood by a Ficoll-Paque density gradient, and isolated PBMCs were diluted in complete medium (consisting of RPMI 1640 [Sigma; containing 2 g/L sodium bicarbonate] supplemented with 20 mmol/L HEPES [Bioshop], 10% fetal bovine serum [FBS; Sigma], and 0.01 mg/mL gentamicin [GIBCO/Invitrogen Life Technologies]). Viable PBMCs were quantified using Trypan blue exclusion (GIBCO/Invitrogen Life Technologies) and a hemocytometer, and 700,000 PBMCs per well were seeded onto the eight-chamber slides (Thermo Fisher Scientific, Lab-Tek II, NalgeNunc) and allowed to adhere for 1 hour (37°C, 5% CO₂). Each time point or condition was tested at least in triplicates and some in quadruplicates if there were enough cells. Media supernatants were aspirated and discarded, and 1% to 1.25% (vol/vol) anti-D–opsonized R₂R₂ RBCs (see below) in complete medium (positive control) was added to the adhered cells for a 2-
hour incubation (37°C, 5% CO₂). Chamber cassettes were then removed, slides were washed in phosphate-buffered saline (PBS) and fixed in 100% methanol, and coverslips added using Elvanol mountant. Elvanol mountant was made in-house using polyvinyl alcohol resin (Sigma), PBS, and glycerin (Invitrogen) (Foo et al, 2007).

2.3.6 Short-term, Overnight Culture of PBMCs using Serum-Containing and Serum-Free Media

Two media were tested for the optimal preservation of monocyte function in MMA: complete RPMI-1640 (Sigma, Missouri, USA) and AIM-V (GIBCO/Invitrogen Life Technologies, Carlsbad, CA). AIM-V is a serum-free, chemically defined medium. To determine if isolated PBMCs that are cultured overnight might be used for MMA, whole blood was mixed with RPMI-1640 or AIM-V media at 1:1 ratio, and PBMCs were isolated using Ficoll-Paque density gradients. PBMCs were recovered and washed three times with phosphate-buffered saline solution (PBS) (Sigma, Missouri, US) and resuspended in designated media in 15mL falcon tubes and stored overnight at 37°C (5% CO₂, humidified). MMA was performed on the short-term cultured PBMCs using complete RPMI-1640 the next day.
2.3.7 Presence of HEPES and/or CO\textsubscript{2} during the MMA

We have previously shown with anti-D–opsonized RBCs that if you use RT for the MMA you do not see phagocytosis but only adherent, rosetting antibody-opsonized RBCs; however, if you use 37°C with a pH buffering system, you see mostly phagocytosis with little adherent RBCs (Rampersad et al., 2005). Whether maintenance of physiologic pH and how best to maintain physiologic pH for MMA results has not been adequately addressed. A parallel comparison was performed to address the importance of pH stability and CO\textsubscript{2} in the MMA, and the effect of ACD and RT storage of whole blood. Complete medium (which contains 20 mmol/L HEPES, see above) or no HEPES were used during the MMA, with half of the samples incubated at 37°C only (with no CO\textsubscript{2}) or a 37°C incubator with 5% CO\textsubscript{2} (to interact with the bicarbonate in the medium). Changes in pH were documented by the color change in the media supernatant (phenol red) and pH test strips (Sigma).

2.3.8 Opsonized R₂R₂ RBCs

R₂R₂ RBCs (from the Blood Collection Center, Canadian Blood Services) in EDTA vacutainer tubes were washed three times with PBS, resuspended in PBS as described previously, and then opsonized with an equal volume of polyclonal anti-D from human serum (Gamma Biologicals/Immucor) for 1 hour with intermittent mixing (37°C, 5% CO\textsubscript{2}) (Foo et al., 2007). Opsonized R₂R₂ were then washed three times with PBS and reconstituted to 1% to 1.25%
vol/vol in complete medium before addition to adhered monocytes. Remaining opsonized RBCs were kept in Alsever's and used in comparison studies at different storage times. In this way the same opsonized R₂R₂ RBCs could be compared to freshly isolated monocyte activity for comparison. We have previously found that opsonization of R₂R₂ cells remains unchanged by indirect antiglobulin test (IAT; 4+) over at least 1 week if stored in Alsever's solution and kept at 4°C.

To test the phagocytic outcome of R₂R₂ RBCs representing weaker IAT strengths, the volumes of polyclonal anti-D were two-fold serially titrated starting from 1:1 vol/vol for opsonization, which corresponded to a decreasing IAT saline tube test scores of 4+, 3+, 2+, 1+ and 0. Same opsonization procedure was utilized as outlined above.

2.3.9 Quantification and Statistical Analyses

Slides were examined by one person throughout the study to minimize reader variability, using phase contrast microscopy (Type DFC345 Fx, Leica Microsystems) as previously described (Rampersad et al, 2005; Foo et al, 2007). At least 200 monocytes per chamber were manually counted and a mean phagocytic index (PRBC; the number of RBCs phagocytosed per 100 monocytes) was reported. Triplicate or quadruplicate data were expressed as mean ± standard error of the mean (SEM) as error bars using computer software (GraphPad Prism, version 5.01,
GraphPad, Inc.). The $t$ test was utilized to compare the mean phagocytic indices. A $p$ value of not more than 0.05 was considered significant.
2.4 Results

2.4.1 Storage of whole blood up to 36 hours preserves monocyte function

Previous studies mentioned that heparinized whole blood can be stored at ambient temperature for up to 36 hours without compromising PBMC phagocytic function; however, no data were provided (Branch et al, 1984). Here, we first examined the phagocytosis of opsonized R₂R₂ RBCs using PBMCs from ACD-anticoagulated whole blood, freshly isolated or after storage at RT for 36 hours (Figure 2.1). Similar levels of phagocytosis were observed with all three donors, whether testing was performed with freshly collected or 36 hours RT-stored whole blood (between 80 and 130 RBCs per 100 monocytes). The degree of phagocytosis observed for Donor 1 was increased slightly after 36-hour storage but was only borderline significant ($p \leq 0.048$).
Figure 2.1 Monocyte phagocytic function after storage of whole blood stored at RT 36 hours.

MMA was performed after whole blood was collected into ACD and compared fresh drawn whole blood to whole blood stored for 36 hours at RT. Three different donors’ results are shown and represent the mean ± SEM, n = 3 to 4. *p ≤ 0.05.
2.4.2 Storage of whole blood up to 48 hours at RT does not preserve monocyte function when compared to freshly isolated PBMCs

To more fully examine the optimal storage time frame, ACD whole blood samples were tested fresh and after 24 and 48 hours of RT storage. The mean phagocytic indices obtained from all three donors were comparable between freshly isolated and 24-hour storage before MMA, with no significant differences (Figure 2.2). However, donor-to-donor variations were observed between 24 and 48 hours of storage before MMA testing. Donor 4 had non-statistically significant differences across the three tested time points, while Donors 1 and 5 had a significant decrease in function by 48 hours (p ≤ 0.0147 and p ≤ 0.0039, respectively; Fig. 2.2).
Figure 2.2 Monocyte phagocytic function after storage of whole blood stored at RT up to 48 hours.

MMA was performed after whole blood was collected into ACD and compared fresh drawn to whole blood stored for 24 and 48 hours at RT. Results of three different donors are shown and represent the mean ± SEM, n = 3 to 4. *p ≤ 0.0147; **p ≤ 0.0039.
2.4.3 Storage of whole blood at 4°C greatly affects PBMC yield

Storage at 4°C of ACD whole blood revealed increased interdonor variation in results, suggesting that some donor monocytes were relatively more resistant to the effects of cold (Donor 1), while others were more susceptible (Donors 4 and 6) as revealed by their dampened phagocytic function (Figure 2.3 A). The only consistent effect of 4°C storage across all the donors tested was that PBMC yield was greatly affected, with a 35% to 40% decrease by 24 hours and 60% to 66% decrease by 48 hours (Figure 2.3 B). In contrast, the PBMC yield fluctuated much less, approximately 12% to 25% by 48 hours, when whole blood was stored at RT.
Figure 2.3 Monocyte phagocytic function and PBMC yield after storage of whole blood stored at RT or 4°C.

A) MMA was performed after whole blood was collected into ACD and compared fresh drawn whole blood to whole blood stored for 24 and 48 hours at RT or at 4°C. Results for three different donors are shown and represent the mean ± SEM, n = 3 to 4. *p ≤ 0.05; **p ≤ 0.01. B) Donors 1 and 6 PBMC numbers after Ficoll isolation from whole blood collected into ACD anticoagulant freshly isolated or after 24 and 48 hours of storage at RT or 4°C.
2.4.4 ACD is the best anticoagulant for MMA

ACD anticoagulant has been favored in our laboratory for the MMA, due to its superior preservative qualities compared to EDTA or heparin (Hess, 2006). However, immunohematology reference laboratories often accept MMA specimens using these other anticoagulants. In this side-by-side comparison, ACD stored samples had the highest mean phagocytic indices in comparison to EDTA and heparin at each time point. Whole blood collected into EDTA or heparin led to a lower phagocytic index using freshly isolated PBMCs, which worsened over time during storage at RT (Figure 2.4 A-C). EDTA or heparin storage was also associated with more rapid and severe decline in monocyte phagocytic function overtime, especially at 48 hours. Finally, storage of the whole blood in either EDTA or heparin led to the presence of lower-density RBC contamination in the Ficoll interface containing the PBMCs at 48 hours, which is suggestive of progressive RBC lesions with storage duration (Figure 2.4 D). In contrast, storage of whole blood in ACD resulted in minimal RBC contamination. These data indicate that ACD best preserves PBMC isolation, without RBC contamination, and monocyte function for the purpose of MMA.
Figure 2.4 Whole blood collected into ACD and stored at RT demonstrates more reliable MMA than whole blood collected in either EDTA or heparin.

(A-C) Results for three different donors are shown. MMA was performed on whole blood drawn into the indicated anticoagulant, using freshly drawn whole blood or whole blood that had been stored at RT for 24, 36, and 48 hours. Results represent mean ± SEM, n = 3 to 4. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. (D) Representative Ficoll density gradients from Donor 4 showing results using whole blood freshly drawn into ACD, EDTA or heparin or after storage for 24, 36, and 48 hours at RT. Results are representative of three different donors. Arrows indicate interface containing PBMCs.
2.4.5 Overnight *ex vivo* cultivation of isolated PBMC using serum-free or serum-containing media led to greatly suppressed monocyte phagocytic function

Next, we examined whether PBMC can be isolated from fresh whole blood and cultivated overnight *ex vivo* before performing the MMA the next day. Since all hematopoietic cells express serum response factor (SRF), a transcription factor involved in the activation of immediate early genes (IEGs) involved in immune cell functions (Halene *et al*, 2010; Taylor & Halene, 2015), we were interested in the effect of serum in the overnight medium used. We compared the use of our regular serum-containing complete RPMI media (with 10% FBS) and a chemically defined serum-free media, AIM-V. Regardless of media used, the overnight incubation of isolated PBMC resulted in significantly depressed phagocytic functions when compared to freshly isolated PBMC, with a slightly more pronounced depression in the serum-free conditions (Figure 2.5).
Figure 2.5 Overnight *ex vivo* cultivation of isolated PBMC using serum-free or serum-containing media led to greatly suppressed monocyte phagocytic function.

Whole blood was collected into ACD anticoagulant and PBMCs were isolated by Ficoll gradient within one hour of collection. PBMCs were then suspended in serum-free media (AIM-V) or serum-containing media (complete RPMI with 10% FBS) and placed into a 37°C incubator with 5% CO₂ and fully humidified. After overnight (O/N) short-term incubation, PBMCs were removed from the incubator and an MMA performed. Results represent mean ± SEM, n=3-4. Statistical comparison between freshly isolated and O/N incubated PBMCs using Student's *t*-test.

*P*≤0.05 ; **P*≤0.01 ; ***P*≤0.001.
2.4.6 Maintenance of physiologic pH is crucial for optimal MMA

The presence of HEPES and 5% CO₂ optimally stabilized the pH within physiologic ranges as evidence by maintenance of phenol red color, and pH strip testing (results not shown), compared to original un-incubated medium (Figure 2.5 A). When HEPES was used alone, without 5% CO₂, the pH was slightly alkaline as seen by the more pinkish color of the medium (pH of 7.5-8.0 by pH strip testing). When there is no HEPES in the medium, but only 5% CO₂, again, the pH becomes slightly alkaline (Figure 2.5 A, right) similar to when there is HEPES but no CO₂. As expected, when there is no pH buffer contained in the medium, this yielded the most alkaline pH, as indicated by the magenta color change (pH of approx. 9 by test strip; Figure 2.5 A, far right). Comparison of MMA using freshly isolated monocytes and monocytes after whole blood stored in ACD for 24 hours yielded no differences across the four conditions tested (Figure 2.5 B). However, despite the maintenance of relative physiologic pH conditions in the presence of HEPES and the absence of 5% CO₂, there was a 34% to 40% reduction in phagocytosis. However, with 5% CO₂ with or without HEPES, the phagocytic function was maintained. A more pronounced depression in phagocytosis (85%-95%) was observed in the absence of both HEPES and 5% CO₂, which also corresponded to an alkaline pH of approximately 9. No adherent RBCs were seen under any conditions examined. Although 5% CO₂ was able to maintain functionality compared to any other condition, the use of HEPES in addition to 5% CO₂ actually seems to maintain a pH as indicated by medium color and pH strip results closer to physiologic pH and may better maintain monocyte functionality in the MMA (Figure 2.5 B); thus, we always use HEPES in our complete medium and a 5% CO₂ incubator for MMA.
Figure 2.6 The maintenance of physiologic pH is crucial for optimal monocyte phagocytic function in the MMA.

(A) Maintenance of pH in the presence or absence of HEPES and/or 5% CO₂ was compared. Relative pH was monitored by the color change of phenol red contained in the medium and confirmed with pH strips (results not shown). (Left three) Complete RPMI medium containing 20 mmol/L HEPES, original un-incubated medium compared to incubation at 37°C with or without 5% CO₂ supplement to exchange with the 2 g/L sodium bicarbonate contained in the medium to buffer the pH. (Right three) Complete RPMI medium without added HEPES, original un-incubated medium compared to medium in a 37°C incubator with or without 5% CO₂ supplement. The more pink to magenta color of the medium indicates an increasingly basic pH.

(B) Results of MMA performed under the different buffer conditions is shown. The absence of both HEPES and CO₂ significantly depressed monocyte phagocytic function. Results from Donor 7 are shown. Results represent mean ± SEM, n = 3. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Black lines and asterisks compared between freshly isolated monocytes, while gray lines and asterisks compared between monocytes from 24-hour-stored ACD whole blood using the t test.
The image shows a comparison of phagocytic index under different conditions. Panel A displays images of test tubes with and without HEPES under 5% CO2 and no CO2. Panel B presents a bar graph comparing the phagocytic index of fresh PBMC and PBMC treated with ACD for 24 hours, under similar conditions. The graph indicates significant differences in phagocytic index between the groups, with asterisks representing statistical significance levels.
2.4.7 Validations of the optimal conditions of ACD and RT storage of whole blood using ten different biological donors

The collection of data presented thus far suggested the optimal storage of whole blood in ACD, at RT for up to 36 hours (Figure 2.1 and 2.4) best preserved monocyte phagocytic functions for the purpose of MMA. We aimed to validate the observations by testing whole blood from seven additional healthy donors, so that in combination with the three previous donors (Figure 2.2), a total of ten different biological donors can be compared to strengthen our claim. Despite the spread of the data generated for each donor due to inter-donor and inter-experimental variations, the overall mean phagocytic indices were comparable between freshly isolated PBMC and PBMC isolated from whole blood after 24 hours storage, and the only significant drop was observed by 48 hours (Figure 2.7).
Figure 2.7 Validations of the optimal conditions of ACD and RT storage of whole blood using ten different biological donors.

The identified optimal conditions of whole blood storage in ACD at RT for up to 24 hours were validated using ten biological donors in different independent experiments. PBMCs from fresh whole blood and whole blood held optimally for 24 hours displayed comparable level of phagocytosis, and a significant drop was observed at 48 hours only. Each data point represents the mean for each donor at each time point, n = 3 to 4. The overall mean phagocytic index ± SEM is represented by the black bars at each time point. Statistical analysis performed using repeated measures two-way ANOVA. **p ≤ 0.01.
2.4.8 Monocyte phagocytosis of opsonized R₂R₂ RBCs of various IAT strengths were maintained from ACD whole blood tested fresh and after whole blood storage in ACD at RT for 24 hours.

The MMA results presented so far were all conducted with maximally opsonized R₂R₂ RBCs that is representative of a strong IAT score of 4+, however, majority of the RBCs sensitization in clinical cases involves weaker DAT and/or IAT. Whether the same optimal storage conditions are still applicable to accurately assess weakly sensitized RBCs was addressed by titrating the anti-D antibody titer used for the \textit{in vitro} opsonization of R₂R₂ RBCs. Two-fold serial dilutions of anti-D antibodies resulted in the corresponding IAT of 4+, 3+, 2+, 1+ and weak reaction (WR). Comparable phagocytosis were obtained across various IAT strengths, with no statistical differences between PBMCs isolated from fresh blood or whole blood after 24 hours storage, although there is slight trend of declining function with storage (Figure 2.8).
Figure 2.8 Monocyte phagocytosis of opsonized R₂R₂ RBCs of various IAT strengths were prepared from ACD whole blood tested fresh and after whole blood storage in ACD for 24-hours. Results from donor 5 are shown. A dose-dependence was observed where the higher IAT strengths corresponded to higher average phagocytic indices. Comparable trends were observed between freshly isolated monocytes and monocytes isolated from whole blood stored for 24-hours, with no statistical differences. Results represent mean ± SEM, n = 3. Statistical comparison between PBMCs isolated from fresh blood and blood after 24-hour storage at RT using Student's t-test. The tests were performed on an additional two donors with similar results.
2.4.9 MMA results from stored and shipped patient whole blood correlates with clinical outcomes

A total of five patient MMAs are presented, four local samples with a storage-matched healthy donor control (Figure 2.9 A-D), and a long-distance couriered sample from Quebec City, which is 800 kilometers away from Toronto (Figure 2.9 E). All whole blood samples were collected into ACD and stored at RT for at least 24 hours before testing in the case of the local samples and up to 30 hours in the case of the Quebec City sample, before processing for the MMA. Clinicolaboratory findings are summarized in Table 2.1.

Table 2.1 Clinicolaboratory results for patients receiving IVIG treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Blood group</th>
<th>RBC source</th>
<th>Hgb (g/L)</th>
<th>DAT</th>
<th>Eluates</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>A+</td>
<td>Pretreatment</td>
<td>153</td>
<td>Negative</td>
<td>NA*</td>
<td>No hemolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post treatment</td>
<td>141</td>
<td>Negative</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>A+</td>
<td>Pretreatment</td>
<td>144</td>
<td>Negative</td>
<td>NA</td>
<td>No hemolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post treatment</td>
<td>137</td>
<td>Negative</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>A+</td>
<td>Pretreatment</td>
<td>139</td>
<td>Negative</td>
<td>NA</td>
<td>Grade 4 hemolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post treatment</td>
<td>119</td>
<td>Positive†</td>
<td>Anti-A</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>AB+</td>
<td>Pretreatment</td>
<td>145</td>
<td>Negative</td>
<td>NA</td>
<td>Grade 4 hemolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post treatment</td>
<td>121</td>
<td>Positive</td>
<td>Anti-A Anti-B</td>
<td></td>
</tr>
<tr>
<td>5‡</td>
<td>Female</td>
<td>AB+</td>
<td>Pretreatment</td>
<td>154</td>
<td>Negative</td>
<td>NA</td>
<td>Grade 4 hemolysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post treatment</td>
<td>74</td>
<td>Positive</td>
<td>Anti-A Anti-B</td>
<td></td>
</tr>
</tbody>
</table>

*NA* = not applicable due to negative DAT.
†Positive DAT with anti-IgG only.
‡Shipped by courier from long-distance site tested within 30 hours of collection into ACD and kept at RT.
The parallel comparison of patient and healthy control MMAs emphasized the importance of autologous over allogeneic PBMCs (Figure 2.9 A-D) as patient and healthy donor mean phagocytic profiles were different, despite the same RBC sources tested, and only the patient PRBC results using autologous monocytes corresponded to clinical outcome. Patients 1 and 2 were clinically free of hemolysis, and indeed their respective phagocytic indices (PRBCs) (black bars) using autologous monocytes were below the cutoff defined by a previous study (Figure 2.9 A and B) (Michelis et al, 2014). However, the healthy donor monocytes (white bars) yielded PRBCs above the cutoff, which would have predicted hemolysis (Figure 2.9 A and B). Patients 3 and 4 both had Grade 4 hemolysis according to the guidelines of the Canadian IVIG Hemolysis Pharmacovigilance Group (Table 2.1; Figure 2.9 C and D). Again, significantly elevated PRBCs in the post sample were observed exclusively with the patient's monocytes and corresponded with the documented hemolysis (Table 2.1), while healthy donor monocytes gave lower PRBCs (Figure 2.9 C and D). The different PRBCs profiles generated from the patient and healthy donor monocytes insinuated differences in immune status, that is, immunosuppression in Patient 2 versus immune activation in Patient 4.

In the long-distance patient sample (Table 2.1, Patient 5), the heightened PRBCs in the MMA correlated with the diagnosis of Grade 4 hemolysis (Table 2.1, Patient 5; Figure 2.9 E). The infused IVIG lot was shown to both opsonize the patient RBCs (Table 2.1) and cause enhanced phagocytic clearance by monocytes in the MMA (Figure 2.9 E). The unopsonized (naive) pretreatment RBCs represented a baseline level of phagocytosis, while the in vitro opsonized pretreatment plus IVIG and in vivo opsonized posttreatment RBCs resulted in higher levels of
phagocytosis, directly correlating the infused lot of IVIG to clinical findings in this patient (Figure 2.9 E).
Figure 2.9 Only the autologous patient MMA, but not the allogeneic healthy control MMA, correlated with clinical hemolysis after IVIG infusion, using stored whole blood.

(A-D) The results shown are from four different patients and healthy controls. Both pre- and posttreatment RBCs were opsonized *in vitro* using a laboratory IVIG lot. (A and B) Patients 1 and 2 did not have hemolysis. (C and D) Patients 3 and 4 were classified as having Grade 4 and 3 hemolysis, respectively, according to the Canadian IVIG Hemolysis Pharmacovigilance Group. (E) Grade 4 hemolysis patient MMA corresponded to clinical outcomes after being shipped more than 800 kilometers from Quebec City to Toronto with a 24-hour courier service. Pretreatment RBCs were not opsonized *in vitro* (baseline), pretreatment plus IVIG opsonized *in vitro* with the infused lot of IVIG associated with hemolysis, posttreatment RBCs were opsonized *in vivo* in the patient. Results represent mean ± SEM, n = 3. Anti-D–opsonized R2R2 RBCs served as a positive control. The dotted line represents the phagocytic cutoff of 17 for IVIG-related hemolysis experimentally determined in a previous publication (Michelis *et al*, 2014).
2.5 Discussion

To date, no comprehensive comparative studies have been conducted to evaluate the optimal conditions under which whole blood should be taken and stored for an MMA, as well as the best assay conditions to optimize the predictive potential of the MMA for studies of auto- and alloantibody clinical significance. Our report is the first to clearly address these issues.

The proposal of storing whole blood at ambient temperature is not a novel idea. Blood banks and biobanks have investigated the effect of overnight holding of whole blood at ambient temperatures to improve the logistics and the quality of downstream processing of blood products (Lu et al, 2011; Eckstein et al, 2015; Dumont et al, 2015). Several studies have shown that overnight holding of whole blood is comparable to fresh whole blood in terms of RBCs, platelet concentrates, and plasma qualities for transfusion purposes (Lu et al, 2011; Eckstein et al, 2015; Dumont et al, 2015). The first suggestion that whole blood for MMA could be stored at RT for up to 36 hours dates back to the 1980s and used heparin anticoagulant (Branch et al, 1984); however, no data were provided in this publication to support the claim. A review publication suggested that whole blood showed no differences in the MMA whether collected into EDTA or heparin, but when stored more than 24 hours at RT, the monocytes lost most of their functionality and after 48 hours, functionality was almost completely lacking (Garratty, 1990). However, this report does not indicate how many different donors were tested and cites only an abstract to support the claim. Because of the paucity of data in the peer-reviewed
literature on this topic, we undertook part of our study to address the issue of anticoagulant and storage conditions for an optimal MMA.

Our results support a previous claim that storage up to 36 hours at RT results in little or no loss of monocyte function (Branch et al, 1984). However, this claim was using heparin anticoagulant, and our findings, using different anticoagulants, which include ACD, are more in agreement with another report that investigated EDTA and heparin, but not ACD, for storage of whole blood for MMA (Garratty, 1990). We agree with this latter report that, when using EDTA or heparin anticoagulant, monocyte function drops off after 24 hours of storage at RT; however, we did not observe as dramatic of a drop-off in activity as reported (Garratty, 1990). Use of ACD anticoagulant, which was not examined in either previous report, maintains monocyte function, similar to freshly tested, over 36 hours when stored at RT (Figures 2.1-2.4 and 2.7). These findings were consistent using multiple donors. We found EDTA anticoagulant to be the least able to maintain optimal monocyte function when compared to ACD or heparin, even when testing freshly isolated monocytes (Figure 2.4). In addition, we find that storage at 4°C over time results in substantial loss of PBMC recovery, corroborating a previous study (Dzik& Neckers, 1984). This decrease has been attributed to apoptosis or necrosis of certain subsets of white blood cells or alterations to cell morphology subject to differential selection during the Ficoll density gradient isolation step (Akriotis & Biggar, 1985). Another effect of EDTA, and heparin, during RT storage was the presence of RBC contamination in the interface containing the PBMCs after Ficoll (Figure 2.4), which was suggestive of alteration in membrane surface area-to-volume ratio and cellular lesions in RBCs (Akriotis & Biggar, 1985). One study examined the potential immunosuppressive effect of in vitro aged RBCs on monocytes and suggested that the
effect was not related to the release of free hemoglobin (Hb), but likely associated with the release of microvesicles (Dzik & Neckers, 1984). This might provide an explanation for our observations of depressed monocyte phagocytic functions with the use of EDTA and heparin during RT storage. Taken together, we do not recommend collecting blood for MMA into EDTA or heparin. We conclude that ACD anticoagulant is the best anticoagulant for maintaining monocyte function, whether tested freshly drawn or after storage up to 36 hours at RT. Although we did not test the anticoagulant citrate-phosphate-dextrose (CPD, blue top tube), as this anticoagulant is formulated to better preserve blood cells than is ACD, we would expect whole blood drawn into CPD to have similar functional outcomes in the MMA as blood taken into ACD, but this should be tested and confirmed in parallel testing.

In addition to the anticoagulants and storage of whole blood, we examined whether MMA could be performed on isolated PBMCs that were short-term cultured and whether this might improve phagocytic function. Ex vivo cultivation of PBMCs has been explored extensively using both serum-containing and serum-free systems (Bennett et al, 1992; Jonuleit et al, 1997; Helinski et al, 1988; Akiyama et al, 1988). For these reasons, we felt it was worthwhile to examine the ex vivo culture of PBMCs overnight in complete medium, with and without FBS. For the MMA, our results clearly indicate that overnight cultivation of PBMCs, regardless of media used, greatly dampens Fc-mediated phagocytosis (Figure 2.5).

Most investigators, as do we, perform the MMA using 37°C in a CO₂ incubator to maintain the physiologic pH between 7.2 and 7.4 (Schanfield et al, 1980; Hunt et al, 1980; Nance et al, 1987; Mendoza et al, 1991; Zupańska et al, 1995). However, some investigators do not control for pH
and/or may even use RT incubation for the assay. In a review article, significant differences in both RBC adherence and phagocytosis were found when MMA was performed with or without culture in a CO₂ incubator, and it was cautioned that an MMA performed in a CO₂ incubator to maintain pH may be important (Garratty, 1990). To answer this question, we have performed side-by-side comparisons marking the benefit of maintaining physiologic pH in the MMA using freshly isolated monocytes or MMA done on whole blood up to 24 hours of storage at RT. Our results clearly show that pH changes rapidly to alkaline when buffers are not present and changes in pH are correlated to depressed monocyte phagocytosis (Figure 2.6). Of interest is that we do not see any adherent RBCs when using these conditions with anti-D, as previously shown (Garratty, 1990). Based on our results, we agree with the review suggesting that use of a CO₂ incubator could be important for optimal results when correlating MMA to clinical outcomes (Garratty, 1990), and recent publications have used a CO₂ incubator (Noumsi et al, 2015; Michelis et al, 2014).

The optimization data presented in Figure 2.1-2.6 utilized maximally sensitized R₂R₂ RBCs using polyclonal anti-D from human serum and represented a strong IAT strength of 4+. However, many clinically relevant antibodies sensitize RBCs at weaker strengths of 1+ or 2+ and nevertheless trigger hemolysis. When we titrated the quantity of anti-D so as to achieve lower IAT strengths with the opsonized R₂R₂ RBCs, a dose-dependent response was observed in monocyte phagocytosis (Figure 2.8). Furthermore, these patterns were nearly identical between freshly isolated PBMCs and PBMCs isolated from overnight-stored whole blood in ACD, with no significant differences (Figure 2.8). This suggests that the overnight whole blood storage at RT maintained the monocyte phagocytic function across a spectrum of opsonization.
For the MMA, it would be desirable to always use patient autologous monocytes in the assay to better reflect what may be happening in the patient. Indeed, it is known that allogeneic monocytes in MMA assays may not provide the correct interpretation of the clinical significance or insignificance of an auto- or alloantibody (Hunt et al, 1982; Gallagher et al, 1983; Branch et al, 1983; Alperin et al, 1983; Branch et al, 1984; Pérez et al, 1986; Branch, D.R., Gallagher, 1986; Mendoza et al, 1991; Zupańska et al, 1995; Michelis et al, 2014; Napier & Rowe, 1987). Despite this, most, if not all, transfusion or reference laboratories that perform MMA for clinical evaluations use fresh whole blood samples from healthy volunteer donors and do not use autologous patient monocytes. This is claimed to be because MMA assays are thought to require immediate attention; thus, the autologous MMA is restricted, as whole blood samples from geographically distant hospital sites cannot be shipped and processed quickly enough. In fact, it has not been well studied as to whether or not samples can be shipped and accurate MMA assay results obtained.

With whole blood drawn into ACD able to be stored at RT for up to 36 hours before processing for MMA, a window is opened to the widely available option of 24-hour courier shipping to reference laboratories. Given individual differences in the immune status of patients having antibodies, because of inflammatory conditions or immunosuppressant therapies such as corticosteroids, the use of autologous monocytes in MMA has been suggested to be a superior approach when trying to determine the significance of auto- or alloantibodies (Hunt et al, 1982; Gallagher et al, 1983; Branch et al, 1983; Alperin et al, 1983; Branch et al, 1984; Pérez et al,
We were unable to investigate the predictive value of our optimal MMA in patients having alloantibodies of a particular degree of known clinical significance due to the unavailability of said samples. However, to illustrate how the MMA under optimal conditions can provide valuable information regarding ability of the assay to predict hemolytic antibodies in patients, we presented five “clinical” MMAs performed after overnight whole blood storage held in ACD at RT. These MMAs were performed on patients that had received IVIG therapy and showed signs of immune-mediated hemolysis caused by the anti-A and/or anti-B IgG antibodies contained in the IVIG product (Michelis et al, 2014). Four patients' MMAs were performed in parallel with a storage-matched healthy control. All four patients' and control samples were drawn into ACD and kept at RT until the MMA was performed after about 24 hours of storage. The differential phagocytic profiles emphasized the importance of autologous over allogeneic PBMCs, with only the autologous MMA corresponding to the observation of hemolysis (Table 2.1; Figures 2.9 A-D). This is likely because the use of patient autologous monocytes takes into account the patient's underlying immune status (existing inflammation or immunosuppressive therapy) and potential genetic polymorphisms of Fcγ receptors (Hunt et al, 1981; Conley et al, 1982; Branch et al, 1983, 1984; Branch, D.R., Gallahger, 1986; Zupanska et al, 1987; Mendoza et al, 1991; Lucas et al, 1993; Zupańska et al, 1995; Arndt & Garratty, 2004). The utility of such optimized storage was highlighted in the sample from a hemolysis patient that was sent to us by courier from 800 kilometer distance, again, with whole blood drawn into ACD and sample kept at RT until tested. The resulting high PRBC of the MMA was consistent with the clinical presentation
of significant hemolysis (Table 2.1; Figure 2.9E). This result strongly suggests that with thermoregulated courier services, autologous patient whole blood can be shipped from distantly drawn sites to laboratories for MMA testing, providing more accurate results for conclusions regarding particular patient antibody clinical significance. Indeed, our elucidation of optimal sample storage conditions in advance of conducting the MMA may expand the perimeter for testing patients and suggests that autologous MMA can be performed on most patients regardless of geographical location as long as samples collected in ACD and kept at RT can arrive within the standard 1-day courier window, thereby enabling the use of autologous monocytes in a most representative manner. However, confirmation of the usefulness of our optimized MMA for predicting which patients having preformed alloantibodies to various RBC antigens may or may not hemolyze when transfused remains to be examined.
Chapter 3

Use of Autologous MMA in Investigating Intravenous Immunoglobulin (IVIG)-Associated Hemolysis

Contents of this chapter present unpublished data that will eventually become part of a more extensive manuscript on the mechanism and predictive factors associated with IVIG-associated hemolysis. This chapter is written with a specific focus on using an autologous MMA in clinical investigations involving stored patient blood samples. All MMA assays were performed by Tik Nga Tong. Patient samples were provided by hospitals within Toronto after informed consent and under individual Hospital REBs.
3.1 Overview

IVIG is an efficacious immunomodulatory therapy for treating a diverse array of autoimmune and inflammatory diseases. However, a subgroup of patients with non-group O blood type receiving high dose IVIG (≥2 g/kg) are at heightened risk for adverse hemolytic reactions. The presence of anti-A and anti-B isoagglutinins within IVIG preparations has been proposed to sensitize and cause FcγR-mediated phagocytosis of non-group O RBCs. In fact, a two-hit model has been previously proposed where pre-existing inflammation is first required to pre-activate the mononuclear phagocytes, so that upon IVIG infusion and sensitization of RBCs, a heightened destruction of RBC results in significant hemolysis. For my thesis, this chapter addresses the clinical applications of an optimized MMA using autologous monocytes (see Chapter 2) by assessing the effect of mononuclear phagocyte activation status on FcγR-mediated phagocytosis of IVIG-sensitized RBC in the high risk subgroup of patients with blood group A, B or AB, and who are receiving ≥2 g/kg IVIG.

3.2 Introduction

IVIG is a blood product that is pooled and purified from thousands of donors’ plasma, consisting of mainly immunoglobulin G (IgG) and traces of IgM and IgA. The use of IVIG has expanded beyond the original treatment of primary immunodeficiency (Imbach et al, 1981), and high dose (≥2 g/kg) IVIG has been used successfully to treat a plethora of autoimmune and inflammatory
related diseases. (Eibl, 2008; Schwartz, 1990; Chipps & Skinner, 1994; Jolles et al, 2005; Guidelines and Recommendations for IVIG). However, despite over 30 years of use in the clinic, the mechanism of IVIG is still not well characterized.

In addition to its clinical efficacy, IVIG has been associated with severe post-infusion hemolytic reactions and the mechanism behind such hemolysis has been gaining research interests to better understand the in vivo function of IVIG and thus better recognize high risk patients to allow early prevention measures (Berard et al, 2012; Berg et al, 2015; Padmore, 2015; Michelis et al, 2014). Indeed, severe cases of IVIG-related hemolysis requiring transfusion compromise patient care and contribute to comorbidity (Berard et al, 2012; Berg et al, 2015; Padmore, 2015; Michelis et al, 2014). A strong ABO bias is observed in which non-group O patients receiving ≥2 g/kg IVIG are at heightened risk for IVIG-associated hemolysis (Berg et al, 2015; Padmore, 2015), which supports the hypothesis that anti-A and anti-B isogglutinins sensitize non-group O RBCs and mediate RBC destruction through complement activation or erythrophagocytosis (Padmore, 2012, 2015; Pendergrast et al, 2015; Bellac et al, 2015). Indeed, it has been well documented that high dose IVIG is often associated with a positive DAT and a drop in hemoglobin that are transient and self-limiting (Berard et al, 2012; Padmore, 2015). However, these observations are not predictive of hemolysis and additional confirmatory laboratory testing are needed as outlined by the Canadian IVIG Pharmacovigilance Group (Devine, 2009).

This simple passive anti-A and anti-B isoagglutinins mechanism does not fully explain the complex etiology, since anti-A and anti-B titers have to be maintained ≤64 under stringent
manufacturer's quality control. Furthermore, not all non-group O patients will hemolyze, despite RBC sensitization, and rare group O patients can hemolyze (Welsh & Bai, 2015; Michelis et al., 2014). Therefore, additional contributing factors must be involved (Padmore, 2012; Pendergrast et al., 2015).

Other observations and hypotheses have been made over the years: soluble ABO antigen secretor status capable of competitive binding to anti-RBC antibodies, IVIG preparation and batch variation with higher anti-A/B titers or proinflammatory contaminants, and Fc gamma receptor (FcγR) polymorphisms capable of enhanced phagocytosis of sensitized RBCs (Kahwaji et al., 2009; Scott & Epstein, 2015; Padmore, 2015; Dhainaut et al., 2013; Kumpel et al., 2003). Perhaps the more widely accepted hypothesis is the two-hit model proposed by Padmore, where clinically significant hemolysis occurs when 1) there is pre-existing inflammation and macrophages are pre-activated with a lower threshold for phagocytosis, and 2) the sensitization of RBC by passive anti-A and B antibodies in IVIG (Padmore, 2012). In support of this model, previous published observations that macrophage activation and elevated serum cytokine interleukin-1 receptor antagonist (IL-1ra), both suggestive of underlying inflammation, are associated with IVIG-related hemolysis in a subgroup of high risk patients of non-group O blood type receiving high dose IVIG (2 g/kg) (Michelis et al., 2014; Pendergrast et al., 2015).

The results to be presented in my thesis represent a continuation of previously published preliminary results (Pendergrast et al., 2015; Tong et al., 2016a) of an ongoing study that will conclude in the Fall of 2016. My role was to further examine the effect of macrophage activation and other serum factors in IVIG-related hemolysis in the same subgroup of high risk patients.
This is an ongoing multicentre project involving patient recruitment from the 5 major hospital sites across Toronto. There are three aims to this project: 1) to identify serum biomarkers that are predictive of clinically significant hemolysis following high dose IVIG, 2) to identify genetic factors that predispose patients as high risk with a focus on secretor status and FcγR polymorphisms, and 3) to evaluate macrophage phagocytic function using the monocyte monolayer assay (MMA) and correlate results to clinical outcomes. The ultimate goal is to characterize and identify factors that would better select high risk patients for close monitoring and preventive measures.

This multicentre collaboration involves transportation of patient samples from hospitals across Toronto to a single Canadian Blood Services site, at the Toronto Centre, for MMA testing. The timely delivery of patient samples poses logistical challenges and renders testing of fresh samples nonviable. Inevitably, samples need to be stored properly during delivery to maintain blood cell function for subsequent assays and permit extraction of useful information from experimental data. As I have determined the optimal conditions of sample collection and storage in Chapter 2, the following data will focus on aim 3 and whether using optimal conditions for MMA correlates to clinical outcome in a subgroup of patients receiving high-dose IVIG therapy.
3.3 Materials and Methods

3.3.1 Patient and Healthy Donor Recruitment

From previous study results, only high risk patients with non-group O blood type receiving high dose \((\geq 2 \text{ g/kg})\) IVIG therapy for their underlying conditions were recruited with REB approval and signed consent (Pendergrast et al., 2015). Another exclusion criteria were patients with underlying anemia and/or background hemolysis prior to IVIG therapy, defined as initial hemoglobin levels below 100 g/L in this study. Careful records of patient weight, blood type and other serological and biochemical results (such as DAT and eluate results, LDH, reticulocytes, indirect bilirubin, haptoglobin, complement, ferritin, etc), underlying conditions, pre-medications, infused IVIG lots, infusion rates and adverse reactions or symptoms were also documented for later analysis of potential correlation. Healthy donors with no known infection or inflammation were recruited as allogeneic controls (Canadian Blood Services Research Ethics Board [REB] approvals 2005.002 and 2005.003).

3.3.2 Sample Collection and Storage

Patient blood samples were collected into ethylenediaminetetraacetic acid (K\(_2\)EDTA; BD) tubes at three different time points and stored at 4°C: baseline sample prior to IVIG infusion (Pre), within 24 hours after last IVIG infusion (Post) and a 5-10 day post follow-up sample (5-10d). Whole blood collected into EDTA was not used for the MMA but only for RBC and plasma
collection. With the last time point collection at 5-10 day post IVIG, an additional blood sample was collected into ACD tubes for subsequent peripheral blood mononuclear cells (PBMCs) isolation and an in vitro functional assay (see Chapter 2). All ACD samples were stored at room temperature for up to 36 hours before testing (see Chapter 2), unless indicated otherwise, due to logistical and geographical reasons of sample delivery. Allogeneic healthy donor blood was collected into ACD tubes only, and was storage-matched with the corresponding patient blood sample for parallel comparisons.

3.3.3 Standard Transfusion Laboratory Testing

Standard transfusion laboratory testing were performed by respective accredited laboratories at Hospital for Sick Children (HSC), St. Michael's Hospital (SMH), Mount Sinai Hospital (MSH), Sunnybrook Hospital (SB) and Toronto General Hospital-University Health Network (UHN) (Brecher et al, 2002). The following blood tests were performed for hemolysis monitoring and confirmatory purposes: direct antiglobulin test (DAT), antibody eluate, hemoglobin (Hb) or hematocrit, white blood cell, platelet and reticulocyte counts, lactate dehydrogenase (LDH), haptoglobin, direct and indirect bilirubin, ferritin, complement proteins (C3 and C4), C-reactive protein and blood smear film. The definition of IVIG-associated hemolysis as outlined by the Canadian IVIG Pharmacovigilance Group, was a drop in hemoglobin (≥10 g/L) and a positive DAT, along with at least two of the following: increased reticulocyte count, LDH, unconjugated hyperbilirubinemia, or low haptoglobin, hemoglobinemia, hemoglobinuria, and/or the presence of significant spherocytosis (Devine, 2009). Blood test results were compared to patient's baseline level and normal observed ranges (Appendix table A1).
3.3.4 MMA

The MMA was performed under optimal conditions as previously published (Tong et al., 2016a; Tong & Branch, 2016) and detailed in Chapter 2.

3.3.4.1 Preparation of Opsonized RBCs

K$_2$EDTA anticoagulated patient blood samples collected over the three different time points and stored at 4°C were used for patient RBCs. Blood were washed three times in PBS and sensitized using a laboratory lot of IVIG (Gamunex) in vitro for 1 hour (37°C, 5% CO$_2$) with intermittent mixing. The amount of IVIG used for in vitro sensitization was calculated based on the patient weight and IVIG received to mimic in vivo sensitization (Appendix table A2). RBCs were washed three times in PBS and reconstituted to 1.25% vol/vol in complete RPMI media.

Anti-D-opsonized R$_2$R$_2$ RBCs are known to yield a potent phagocytic response, thus were used as a positive control to ensure the MMA was performed correctly and to also evaluate differences in macrophage activation. R$_2$R$_2$ RBCs (obtained from the Blood Collection Center, Canadian Blood Services) in K$_2$EDTA vacutainer tubes were washed three times in PBS and opsonized with 6:4 vol/vol with polyclonal anti-D from human serum (Gamma Biologicals/Immucor) for 1 hour (37°C, 5% CO$_2$) with intermittent mixing. After 1 hour of in
*vitro* sensitization, all RBCs were washed three times in PBS and reconstituted to 1.25% vol/vol in complete RPMI media.

### 3.3.4.2 Phagocytosis

After 1 hour of incubation, the monocyte-macrophage population within the PBMC had adhered to the chamber, while other non-adherent lymphocytes were removed by discarding the supernatant. The reconstituted *in vitro* sensitized RBCs were then added to the monocyte monolayer and incubated for 2 hours (37°C, 5% CO₂) for FcγR-mediated phagocytosis.

### 3.3.5 Quantification and Clinical Correlations

Slides were examined using phase contrast microscopy (Type DFC345 Fx, Leica Microsystems) as previously described (Michelis *et al.*, 2014; Tong *et al.*, 2016; see Chapter 2 of the thesis). At least 200 monocytes per chamber were manually counted and a mean phagocytic index (the number of RBCs phagocytosed per 100 monocytes) was reported. Triplicate data were expressed as mean ± standard error of mean (SEM) as error bars using computer software (GraphPad Prism, version 5.01, GraphPad, Inc.). MMA data were correlated to clinical outcomes of patients, using an experimentally determined cut-off of 17, above which would be considered a clinically significant MMA readout (Michelis *et al.*, 2014).
3.4 Results

Serological results from hospital laboratories and MMA results from Canadian Blood Services from a total of 26 patients are presented in Table 3.1.
Table 3.1 Complete tabulated patient serological and MMA investigations in high dose IVIG-associated hemolysis.

<table>
<thead>
<tr>
<th>Patient</th>
<th>ABO/Rh</th>
<th>Hemolysis status</th>
<th>RBC samples</th>
<th>Hbg (g/L)</th>
<th>DAT</th>
<th>Anti-IgG</th>
<th>Eluate</th>
<th>LDH</th>
<th>Total/ indirect bilirubin</th>
<th>Retics (10^3/L)</th>
<th>Haptoglobin (g/L)</th>
<th>MMA Results</th>
<th>Clinical Correlation</th>
<th>Sample Storage</th>
<th>Special Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>UHN-01</td>
<td>A+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>&lt;24 hour at RT</td>
<td>No healthy control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pre</td>
<td>115</td>
<td>Neg</td>
<td></td>
<td>157</td>
<td>6/6</td>
<td>64</td>
<td>0.47</td>
<td>1.15±0.38</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post</td>
<td>101</td>
<td>Neg</td>
<td></td>
<td>136</td>
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**SB-02** AB+ Grade 4

- Pre 124 Neg
- Post 115 Pos (1+)
- 5-10d 70 Pos (1+)
- R,R_2 (ctrl) 13.50±3.42

**UHN-15** A+ Grade 4

- Pre 124 Neg 142 6/6 54 2.34
- Post 112 Pos (WR) Pos Anti-A 157 4/2 51 1.83
- 5-10d 121 Neg 152 7/5 88 1.83
- R,R_2 (ctrl) 3.68±0.48

**SB-03** A+ Grade 2

- Pre 152 Neg 197 8/8 2.4
- Post 140 Pos Pos 187 16/7 33 1.6
- 5-10d 143 Neg Anti-A 210 14/9 76 1.63
- R,R_2 (ctrl) 1.30±0.29

**UHN-19** A+ Grade 2

- Pre 142 Neg 223 57/40 53 3.2
- Post 122 Neg 179 68.2/58.5 122 1.21
- 5-10d 112 Pos (1+) Pos Other 186 38/30 244 0.4
- R,R_2 (ctrl) 35.23±7.03

**SB-04** B+ Grade 2

- Pre 133 Neg 177 6/7 83 1.2
- Post 112 Pos (1+) IgG Other 130 8/7 57 0.89
- 5-10d 126 Neg 135 7/7 100 1
- R,R_2 (ctrl) 70.44±1.00

**MSH-02** A+ Grade 2

- Pre 115 Neg 309 6/4 60 0.76
- Post 103 Pos (WR) Pos Anti-A 224 5/62 76 0.72
- 5-10d 116 Neg 310 8/6 99 0.46
- R,R_2 (ctrl) 53.35±5.06

**SMH-02** A+ Grade 2

- Pre 115 Neg 309 6/4 60 0.76
- Post 103 Pos (WR) Pos Anti-A 224 5/62 76 0.72
- 5-10d 116 Neg 310 8/6 99 0.46
- R,R_2 (ctrl) 53.35±5.06

**SB-02** AB+ Grade 4

- Pre 124 Neg
- Post 115 Pos (1+)
- 5-10d 70 Pos (1+)
- R,R_2 (ctrl) 13.50±3.42

**UHN-15** A+ Grade 4

- Pre 124 Neg 142 6/6 54 2.34
- Post 112 Pos (WR) Pos Anti-A 157 4/2 51 1.83
- 5-10d 121 Neg 152 7/5 88 1.83
- R,R_2 (ctrl) 3.68±0.48

**SB-03** A+ Grade 2

- Pre 152 Neg 197 8/8 2.4
- Post 140 Pos Pos 187 16/7 33 1.6
- 5-10d 143 Neg Anti-A 210 14/9 76 1.63
- R,R_2 (ctrl) 1.30±0.29

**UHN-19** A+ Grade 2

- Pre 142 Neg 223 57/40 53 3.2
- Post 122 Neg 179 68.2/58.5 122 1.21
- 5-10d 112 Pos (1+) Pos Other 186 38/30 244 0.4
- R,R_2 (ctrl) 35.23±7.03

**SB-04** B+ Grade 2

- Pre 133 Neg 177 6/7 83 1.2
- Post 112 Pos (1+) IgG Other 130 8/7 57 0.89
- 5-10d 126 Neg 135 7/7 100 1
- R,R_2 (ctrl) 70.44±1.00

**MSH-02** A+ Grade 2

- Pre 115 Neg 309 6/4 60 0.76
- Post 103 Pos (WR) Pos Anti-A 224 5/62 76 0.72
- 5-10d 116 Neg 310 8/6 99 0.46
- R,R_2 (ctrl) 53.35±5.06

**SMH-02** A+ Grade 2

- Pre 115 Neg 309 6/4 60 0.76
- Post 103 Pos (WR) Pos Anti-A 224 5/62 76 0.72
- 5-10d 116 Neg 310 8/6 99 0.46
- R,R_2 (ctrl) 53.35±5.06
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<td>Sample Storage</td>
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*DAT direct antiglobulin test
†LDH Lactate dehydrogenase
‡WR weak reaction

Immune suppressed (prednisone, azathioprine, methotrexate, and hydroxychloroquine)
3.4.1 *In vivo* sensitization of RBCs with passive anti-A and anti-B does not always result in enhanced destruction and hemolytic outcome

Nine of the 26 patients were confirmed with a diagnosis of IVIG-associated hemolysis of various degree (Devine, 2009) depending on the drop in Hgb level, with three Grade 1 (Hgb drop of 10-20 g/L), three Grade 2 (Hgb drop of 21-30 g/L) and three Grade 4 (Hgb drop of >40 g/L) (Table 3.1). Analysis of serological test results revealed that no single test was sufficient to confidently distinguish patients with hemolysis from those without (Table 3.2). Interestingly, even with a positive DAT, a Hb drop of >10 g/L and the presence of passive anti-A and/or anti-B in the eluate, this was not adequate to confirm hemolysis, since seven patients fulfilled these criteria with no clinical manifestation of hemolysis (UHN-06, UHN-15, SB-03, UHN-19, SMH-02, SMH-03 and SB-09) (Table 3.1).
Table 3.2 Summary of standard serological results and correlations with clinical outcomes of hemolysis.

<table>
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<th>Patients with hemolysis (9)</th>
<th>Patients without hemolysis (16)</th>
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<td>9/9 (100%)</td>
<td>13/16 (81%)</td>
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<td>Positive DAT</td>
<td>9/9 (100%)*</td>
<td>8/16 (50%)†</td>
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<tr>
<td>Eluate with anti-A and/or anti-B</td>
<td>8/9 (89%)</td>
<td>7/16 (44%)</td>
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<td>Increase in LDH (50% from baseline)</td>
<td>4/9 (44%)</td>
<td>0/16 (0%)</td>
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<tr>
<td>Unconjugated hyperbilirubinemia (50% increase from baseline)</td>
<td>4/9 (44%)‡</td>
<td>4/16 (25%)§</td>
</tr>
<tr>
<td>Increase in reticulocyte counts (50% increase from baseline)</td>
<td>8/9 (89%)</td>
<td>4/16 (25%)</td>
</tr>
<tr>
<td>Low haptoglobin level (50% decrease from baseline)</td>
<td>8/9 (89%)</td>
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</table>

*Three weak reactions, five 1+, and one 2+
†Three weak reactions, three 1+, and two 2+
‡Associated with all severe Grade 4 and one Grade 2 hemolysis
§Three were associated with compensatory production of reticulocyte
||One patient with missing data and thus cannot be assessed
¶Four patient had a 40% drop
3.4.2 Use of MMA to investigate the phagocytic destruction of patient RBC
correlated well with clinical outcome of hemolysis, especially when
autologous patient PBMCs were used

Next, the MMA was used to evaluate the FcγR-mediated destruction of patient RBC sensitized
by passive anti-A and anti-B with IVIG. Although the post and 5-10d patient RBCs had been
sensitized *in vivo* by the infused IVIG therapy, all the patient RBCs (Pre, Post and 5-10d) were
also sensitized *in vitro* using a laboratory lot of IVIG to ensure sufficient antibody coating just
prior to the MMA. A total of 26 patients and 24 healthy controls were examined using the MMA,
however two patients had missing RBC samples (SB-02 and MSH-04) and hence were not
included in further analysis (Figure 3.1).
Figure 3.1 Clinical correlations based on MMA results.
Clinical correlations were evaluated based on an experimentally determined cut-off of 17 previously published in a similar study (Michelis et al, 2014). Of the remaining 24 patient MMA and 22 healthy control MMA, 18/24 (75%) were correlative and 6/24 (25%) were not. Taking a closer look at the correlating cases in Figure 3.1, 10/18 (42% of total) were achieved only when patient PBMCs were used in the MMA, while the other 8/18 (33% of total) were obtained with both patient and healthy control PBMCs. Two of the 10 clinically correlating patient MMA (UHN-01 and MSH-01) were due to the lack of parallel healthy controls and another one exhibited an unexplained enhanced destruction of Pre-IVIG RBC (UHN-15). For the reminder seven cases, five can be attributed to immunosuppression (UHN-06, UHN-07, UHN-12, SB-03, UHN-19) and two to immune activation (UHN-31 and UHN-34) in patients.

The anti-D sensitized R₂R₂ RBCs served as a control known to elicit a potent phagocytic response, and hence were used to infer immune suppression or activation between patient and healthy control PBMC. A drastic decrease in phagocytosis was observed for patients UHN-06, UHN-12, SB-03, UHN-19 when compared to healthy controls (8.07 ± 1.7 vs. 60.92 ± 1.8, 5.63 ± 0.96 vs. 29.23 ± 3.22, 6.98 ± 1.82 vs. 19.86 ± 4.70, 1.30 ± 0.29 vs. 25.54 ± 0.91, respectively) (Table 3.1 and Figure 3.2A-D). Immunosuppression of patient UHN-06 and UHN-12 could be ascribed to pre-medication with tacrolimus plus azathioprine, and prednisone, respectively (Table 3.1). On the other hand, enhanced patient macrophage phagocytic functions correlated with active hemolysis in UHN-31 and UHN-34 as denoted by the black arrows (Figure 3.2E and F).
Figure 3.2 The use of autologous PBMCs reveals immune suppression and/or activation in patient monocyte phagocytic function and thus correlates better with clinical outcomes of IVIG-associated hemolysis.

(A-D) Patient monocytes had significantly decreased phagocytic indices (black bars) in comparison to the healthy control (white bars), despite the same IVIG-sensitized patient RBCs (Pre, Post, 5-10d) or anti-D sensitized control R₂R₂ RBCs being tested. (E and F) Immune activation of patient monocyte function as revealed by the heightened phagocytosis that correlated with clinical symptoms during active hemolysis of Grade 4 severity (black arrows), but were not evident when allogeneic healthy PBMCs were used. Results represent mean ± SEM, n =3. The dotted line represents the phagocytic cut-off of 17 for IVIG-related hemolysis experimentally determined in a previous publication (Michelis et al, 2014).
3.4.3 Use of the MMA might not be suitable in assessing low-grade IVIG-associated hemolysis

There were 6/24 (25%) non-correlating MMA results, with five false negatives and one false positive. The single false positive was observed with MSH-02, where the heightened phagocytic indices (mean ± SEM) for Pre and Post of 51.44 ± 2.66 and 65.31 ± 2.68, respectively, would have predicted severe hemolysis. However, other than a hemoglobin drop of 21 g/L and a positive DAT, other blood work results were not indicative of RBC hemolysis (Table 4). The five negative cases were associated with low grade hemolysis, with two Grade 1 (UHN-33 and SMH-01c) and three Grade 2 (SB-04, UHN-28 and SB-11). SMH-01c had suppressed phagocytic function that was likely associated to prednisone administration, whereas SB-04 was diagnosed with delayed reaction secondary to IVIG. UHN-28 and SB-11 patient ACD blood samples were stored at room temperature for 48 hours prior to PBMC isolation, and hence might attribute to potential suppression of phagocytic functions (Tong et al, 2016 and Chapter 2).
3.5 Discussion

(Note: Although the broader goal of the described study was to investigate the mechanism of IVIG-related hemolysis, for the uniformity of this thesis, the discussion will focus on the clinical application of the MMA and the relevance of use of autologous patient samples even after storage.)

With the increasing demand of IVIG therapy, there is an increased interest in preventing and managing the adverse reactions associated with the therapy. One of the adverse reactions is hemolysis, where symptoms are typically mild and self-limiting as IVIG is metabolized and cleared from the circulation, but there are infrequent cases of acute severe hemolytic reactions, where some required emergency RBC transfusion to stabilize the hemoglobin level (Kahwaji et al., 2009; Welles et al., 2010; Berard et al., 2012; Michelis et al., 2014; Berg et al., 2015; Welsh & Bai, 2015). Currently, there are no preventive measures available and the management of hemolysis is mostly supportive care (Welsh & Bai, 2015).

Similar to the immunomodulatory mechanism of IVIG, the etiology of IVIG-related hemolysis is not well characterized. The two identified major risk factors, non-group O blood type and high doses of IVIG (≥2 g/kg), support the hypothesis that passive anti-A and anti-B isogglutinins within IVIG sensitize non-group O RBCs in the recipient and causes FcγR-mediated destruction of RBC (Padmore, 2012, 2015; Pendergrast et al., 2015; Bellac et al., 2015). The data presented
herein aim to evaluate the clinical utility of MMA in investigating the contribution of anti-A and anti-B in IVIG-related hemolysis and assess the potential advantage of autologous over allogeneic PBMC.

The MMA was originally developed to assess and predict the clinical significance of anti-RBC alloantibodies in mediating hemolytic transfusion reactions (Schanfield et al, 1980; Hunt et al, 1980; Branch et al, 1984). By evaluating in vitro FcγR-mediated phagocytic destruction of alloantibody sensitized RBCs, the in vivo survival of RBCs can be inferred. By exploiting the same mechanism involved in extravascular hemolysis, the MMA was recently modified to investigate the potential involvement of passive anti-A and anti-B in IVIG-related hemolysis (Michelis et al, 2014; Pendergrast et al, 2015). In the pioneering publication by Michelis et al, a rare Grade 4 hemolysis case was reported in an ABO-mismatched (recipient O, donor A) marrow transplant patient upon high-dose IVIG therapy to treat immune thrombocytopenia (ITP) (2014). Both standard reference laboratory blood testing and MMA investigations revealed multiple contributing factors to the severity and rarity of this case: 1) overestimated IVIG dosing based on unadjusted weight in obese patient, 2) differential A antigen expression due to chimerism, 3) pre-existing inflammation with elevated pro-inflammatory cytokines (IL-1ra and IL-1β) before IVIG administration, and 4) heightened phagocytic activity when patient mononuclear phagocytes were incubated with patient's own RBCs, but not with reference type A1 cells (Michelis et al, 2014). The above evidence supports the proposed "two-hit" model of IVIG: first there was a pre-existing inflammation status at time of IVIG infusion, and then the unadjusted IVIG dosing and different A antigen expression led to enhanced sensitization and thus destruction of RBCs (Padmore, 2012; Michelis et al, 2014). Most importantly, the MMA
results provided evidence that IVIG-mediated hemolysis is a complex interplay between patient's mononuclear phagocytes and IVIG-sensitized autologous RBCs, that cannot be mimicked by the use of allogeneic mononuclear phagocytes and unrelated reference blood typed cells.

The current study provides additional data to support the more relevant use of autologous patient samples in correlating the MMA to clinical outcomes. With a total of 26 patient MMAs, there were 18 clinically correlating and 6 non-correlating cases, and the remainder 2 were excluded from further analysis due to missing blood samples. Although 8 out of 18 correlating cases were obtained from both autologous and allogeneic PBMCs, the reminder 10 were obtained only when using autologous patient PBMC, and 7 of these could be attributed to immune suppression or activation inferred from mononuclear phagocyte function. This is likely related to the fact that allogeneic cells cannot recapitulate the mononuclear phagocyte activation status and potential FcγR polymorphisms present in the patient, both of which may affect phagocytic outcomes (Padmore, 2012; Kumpel et al, 2003; Pendergrast et al, 2015).

The use of MMA is not perfect in clinical correlations, with six non-correlating cases. Four of the six cases could be explained by sub-optimal storage of blood samples, hemolysis unrelated to IVIG therapy and the administration of immunosuppressants. In fact, five out of the 24 patient MMA results cannot be explained using the serology data, patient demographics and/or medication records. There are several potential reasons. First, the cut-off of 17 for clinical significance was empirically determined based on the average (± standard deviation) phagocytic outcome against IVIG-opsonized A1 RBCs by 21 different healthy donors as previously
described (Michelis et al, 2014). Because this publication is the only published study on IVIG-related hemolysis investigations using the MMA, the published cut-off was adopted for the analysis of this study. When the entire study is completed, the cut-off will have to be re-evaluated and may be changed to better represent the current cohort of patients. Second, other FcγR-independent mechanisms of RBC destruction might be involved but are not assessed by the MMA, such as complement-mediated RBC destruction, although to date complement has not been directly implicated in IVIG-associated hemolysis. Studies on IVIG-mediated hemolysis have suggested the potential contribution of scavenger receptors on mononuclear phagocytes removal of RBC (Liepkalns et al, 2012; Terpstra & van Berkel, 2000; Pendergrast et al, 2015). Whether the MMA can assess scavenger receptor mediated phagocytosis has not yet been evaluated, although IgG-sensitization of RBC seems to be crucial as shown in baseline phagocytosis of IVIG-unsensitized RBC when compared to heightened level of phagocytosis of IVIG-sensitized RBC (Michelis et al, 2014). Third, the use of a laboratory lot of IVIG for in vitro sensitization of patient RBC as opposed to the original infused lots represents one major caveat of the study design. Variations in different IVIG preparations have been documented and certain adverse reactions can be back tracked to a single suspected IVIG lot (Dhainaut et al, 2013; Michelis et al, 2014; Scott & Epstein, 2015). Currently, there are no guidelines to keep IVIG aliquots in storage for retrospective investigations upon adverse incidents, hence there is no way to retrace the implicated IVIG lot once it has been depleted. Lastly, the observed clinical inconsistency might stem from the diversity in patients' underlying pathologies that require additional clinical data for a more complete assessment.
Another important implication of this study relates to the logistics of clinical sample transport. The convention to use fresh blood samples for MMA, especially when blood cells are used in subsequent functional in vitro assays, is based on the assumption that cellular functions diminish with ex vivo storage. This poses challenges for clinical and basic research collaborations that rely on the timely delivery of patient samples between geographically distant hospital sites and testing laboratories. A previous publication examined the optimal storage conditions of blood samples found that the combined use of ACD anticoagulant at room temperature for up to 36 hours preserves mononuclear phagocytes' function in FcγR-mediated phagocytosis as assessed by the MMA (Tong et al, 2016a and Chapter 2 of my thesis). However, the optimization was performed mostly on healthy donor blood with a few clinical examples (Tong et al, 2016a). Whether pre-existing inflammation in patients would have detrimental effects on phagocyte function during the same optimized storage conditions requires further investigations. Due to both the geographical distance and time of blood draw, the timely delivery of clinical samples within 2 hours of blood draw for "fresh" processing was deemed impossible. Indeed, only 1 out of the 26 patient samples was processed “fresh”, while other blood samples were stored under optimal conditions (Chapter 2 and table 3.1). Despite the ex vivo storage, 18 clinical samples yielded clinically correlating results. This provides additional support that the processing of clinical blood samples can be delayed for the MMA when they are stored under optimal conditions and can yield clinically relevant data.

In conclusion, the preliminary results presented herein support the "two-hit" model, where macrophages are activated prior to receiving anti-A/-B in the high-dose IVIG, resulting in heightened phagocytic destruction of IVIG-opsonized RBCs and hemolytic reactions. Results
from cytokine, microparticles, ABO zygosity and secretor genotyping, and FcγR expression and polymorphism analyses from the same cohort of patients will provide further insights into the complex mechanism of IVIG-associated hemolysis. In addition, although it may be most ideal to use fresh blood samples, the delayed processing of clinical blood samples for the purpose of MMA did not compromise the relevance of data generated, especially when samples were stored properly as recommended by Tong et al (2016a). Further investigations with a larger cohort of patients that have auto- or alloantibodies to RBC antigens and require transfusion of donor blood having the corresponding antigen(s) are needed to validate such storage conditions in a reference laboratory setting.
Chapter 4

Overall Discussion, Conclusions and Future Directions
4.1 Overall Discussion

The MMA has been used for more than 35 years for research purposes and has been used by a few laboratories for the clinical assessment of transfusion compatibility in the United States. My thesis is novel as it is the first comprehensive examination of anticoagulant use, storage conditions, and the role of pH for optimal assay conditions to be reported. It is also the first study to assess whether blood samples can be delayed in processing while maintaining their clinical relevance, which is important if autologous patient samples are to be examined. A search through the published literature revealed that storage conditions of blood samples for the MMA have been largely undiscussed (Hunt et al, 1980, 1981; Nance et al, 1987; Lucas et al, 1993), or that different sets of conditions were used for different publications (Branch et al, 1983, 1984; Garratty, 1990; Zupańska et al, 1995; Noumsi et al, 2015; Michelis et al, 2014). The most recent extensive review on the technical aspects of MMA dated back to 1990, where the freshness of monocytes, effects of anticoagulant, assay incubation atmosphere, sensitivity readout of adhesion versus phagocytosis were discussed (Garratty, 1990). However, supporting data were either referenced from an abstract and thus not peer reviewed, or unpublished observations that were not shown. To address these discrepancies and a lack of peer reviewed data and consensus observed in the literature, I sought to optimize the sample storage and assay conditions that would maintain the monocyte phagocytic functions for the purpose of MMA. I used a systematic approach that identified the combination of whole blood storage in ACD anticoagulant, at room temperature between 18-22°C, and up to 36 hours best preserve the monocyte phagocytic function when compared to freshly processed samples (Chapter 2). The first observation that
patient blood samples could be stored prior to MMA dated back to 1984, where whole blood in heparin stored at RT for up to 36 hours still correlated to clinical outcome, but no parallel comparisons data were provided (Branch et al, 1984), only a statement that this was the case. Two years later, a published abstract reported that whole blood in EDTA or heparin at RT for up to 48 hours resulted in greatly reduced monocyte phagocytosis, with decline in activity observable at 24 hours (Garratty et al, 1986). Again, no data were provided with the claim. In this thesis, I tested the three common anticoagulants and found that ACD best preserved monocyte phagocytic function (Chapter 2, Figure 2.4). This observation is consistent with the literature, where the storage of whole blood in ACD resulted in stable light scatter cell populations when compared to fresh whole blood (Thornthwaite et al, 1986; Mallone et al, 2011).

Another controversy is related to assay incubation conditions. Previously published MMA readouts can be divided into three major types: 1) RBC adherence (reactivity defined as ≥3 rosetting RBC), 2) phagocytosis, and 3) a mixture of adherence and phagocytosis. The different types of reactivity observed were later found to be due to assay incubation conditions, where both 37°C and 5% CO2 are needed for phagocytosis (Gallagher et al, 1983; Branch et al, 1984; Wren & Issitt, 1986), and the absence of either would lead to mostly RBC adherence with little phagocytosis (Nance et al, 1987; Hunt et al, 1982). Indeed, similar observations were made when I tested the presence and/or absence of 5% CO2 and/or HEPES supplement, and the reduced phagocytosis was associated with the alkalinity of media (Chapter 2, Figure 2.6). Both the use of 5% CO2 and HEPES buffer are used to maintain physiologic pH at 37°C. Therefore, our data provided strong evidence that the maintenance of physiological pH is crucial for the
MMA (Chapter 2). My results put to rest any controversy regarding maintaining physiological pH and temperature of 37°C when performing the MMA for optimal results. This is further reinforced in the clinical correlations with patient samples.

In addition to optimal storage recommendations mentioned above, I discourage the use of EDTA or heparin anticoagulant, 4°C storage, and the prolonged incubation of isolated PBMCs, where significantly depressed mononuclear phagocyte functions, decreased cellular yield or heightened inter-donor variability were observed (Chapter 2). Although surface markers and serum factors were not examined as part of my study, I speculate the decline in function to be due to a combination of granulocyte activation (Mallone et al, 2011), the release of microvesicles from stored RBCs (Levin et al, 2013; Prudent et al, 2015; Wisgrill et al, 2016) and/or cytokine production (Hodge et al, 2005).

I then sought to address whether the optimal conditions established from healthy donor blood can be applied to patient blood where there is background inflammation. Due to the fact that we are not a reference laboratory, we do not receive patient samples for pre-transfusion compatibility testing on a regular basis. Over the past two years, a total of seven blood samples from four "difficult to transfuse" patients (Appendix Table A3), including one published case study (Tong et al, 2016b), were sent to our laboratory for the MMA crossmatch testing. However, none of the patients were actually transfused, rendering clinical predictive correlations infeasible. Also, all of the case studies were strategically timed, so that patient blood samples were tested fresh within 2
hours of blood draw, therefore I was not able to validate the storage conditions using these patient samples.

Although clinical samples for predicting HTRs are difficult to come by, I am involved in a multicentre study investigating the mechanism of IVIG-associated hemolysis in a group of high risk patients receiving high dose IVIG (>2 g/kg) for their underlying inflammatory diseases. The constant recruitment of patients represents a steady supply of clinical samples. Since delays in sample delivery are inevitable due to multicentre involvement and with the knowledge of the optimal storage conditions, we started requesting samples to be kept at room temperature until delivery and holding samples that are delivered late in the afternoon at room temperature until testing in the next morning. With the use of MMA, I assessed FcγR-mediated phagocytic destruction of patient RBCs due to IVIG sensitization. Within the preliminary data presented in Chapter 3, only one out of 26 patient MMA was processed fresh. Despite the delay in sample processing, 75% (18/24) of the results correlated to clinical outcomes, and 42% (10/24) correlations were obtained using autologous patient samples only (Chapter 3). Indeed, the MMA is not perfect, with 25% (6/24) clinically non-correlating results. This provides evidence that the optimal storage conditions (Chapter 2) can be applied to patient blood samples, while yielding clinically correlative results, and also supports the use of autologous over allogeneic blood samples for more relevant results. Although the majority (4 out of 6) of the non-correlating cases can be attributed to known factors (e.g. sub-optimal storage for 48 hours, delayed hemolytic reactions unrelated to IVIG therapy, and immunosuppression due to corticosteroid administration), the remaining two cannot be explained within the realm of available data. This represents the limitation of the MMA in evaluating IgG and FcγR-dependent destruction of
RBCs and thus predicting the extent of extravascular hemolysis only. Indeed, the clinical severity of HTRs is related to both the rate of RBC destruction (both intra- and extravascularly), and the rate of compensatory reticulocytosis *in vivo* (Barcellini, 2015; Kaushal *et al.*, 2016). Despite the physiological mimicry of FcγR-mediated phagocytosis in this *in vitro* functional assay, the MMA is far from replicating the complexity of *in vivo* hemolysis.

The use of blood samples from patients who received IVIG therapy in a retrospective study to validate the optimal storage conditions intended for predictive clinical assessments of HTRs is not the ideal parallel comparison. The respective MMAs have different opsonization procedures and different clinically significant cut-offs, where the former tests phagocytic response to anti-A and anti-B sensitized autologous RBCs, and the latter tests autologous serum antibodies sensitized allo geneic RBCs. Despite the differences, the MMA is still used to assess the FcγR-dependent phagocytic destruction of RBCs as a means to infer *in vivo* extravascular hemolysis. The high clinical correlations obtained from stored patient blood despite the background inflammation provides convincing evidence that the optimal storage conditions can be applied where immediate processing and testing of clinical samples cannot be accomplished.

Recently, several clinical collaborators expressed interest in promoting and validating the use of MMA in accredited reference laboratories within Canada (personal communication, Dr. DR Branch). Indeed, when dealing with difficult to transfuse patients, such as those with alloantibodies against a high prevalence antigen, or multiple auto/alloantibodies, the current pre-transfusion compatibility testing scheme is time consuming to identify the specificity of the
antibody and to locate an antigen-negative unit. In addition, the serological crossmatch of almost 100% matching blood still resulted in HTRs in certain patients (Walker et al, 1965; Gupta et al, 2015; McGlennan & Grundy, 2005), further exemplifying the notion that hemolytic reactions are more complex than the simple presence of antibody and antigen-positive blood. The niche of the MMA is to supplement the current crossmatching scheme by determining the significance of the suspected antibody or antibodies in mediating RBC destruction via FcγR-mediated phagocytosis. The MMA is uniquely suited for this due to the use of autologous patient mononuclear phagocytes to more accurately predict the in vivo survival of transfused RBC, due to the inclusion of FcγR polymorphisms and current immune status of patient.

In setting up the MMA as a routine reference laboratory test, already optimized conditions are required for the test to be most predictive of auto- or alloantibody clinical significance. In my thesis, I have investigated the optimal conditions required for the MMA and shown that using these conditions provides for a reasonable predictive value of the assay. Although further testing is required with patients having various antibodies to RBCs requiring transfusion, these tests are currently being performed in Dr. Branch’s laboratory (Tong et al, 2016a).

Major obstacles in setting this assay up in reference laboratories are (1) the proper handling of patient blood samples during shipping between sites of blood draw in clinics or hospitals to the site of laboratory testing. (2) The MMA is a 6-8 hours long in vitro assay that requires final manual readout using a microscope. (3) Performance of the MMA requires, highly trained lab personnel with the appropriate biosafety training and adequate experiences in tissue culture. If
MMA was to be set up in Canada, most probably, it would require one to two centralized testing facilities, one in Eastern and one in Western Canada. In my thesis, I have shown that use of autologous monocytes is possible if optimal conditions of anticoagulant and storage temperature are maintained. Indeed, samples stored at RT over 24 hours or shipped for testing from over 800 km distance, by 24 hr courier gave correlative results with immune hemolysis (Chapter 1). Despite the availability of 24-hour courier and/or air services, with the administrative work of sample release and the landmass of Canada, it is inevitable for delayed blood samples testing. Thus, there is still a potential need for healthy allogeneic donors in the MMA. Recently, investigators from Edmonton have shown that PBMCs from multiple healthy donors can be pooled, cryopreserved, and then thawed and reconstituted when needed (J. Holovati, personal communication to Dr. DR Branch). Using these pooled cryopreserved and thawed PMBCs, it was shown to give similar results in the MMA with control, opsonized cells and may be a viable alternative to using autologous monocytes or freshly isolated normal, healthy donors if correlated to transfusion outcomes.
4.2 Conclusions

The utility of the MMA for research and clinical diagnostic purposes has been well documented in the literature and the results presented herein. In Chapter 2, I addressed the discrepancy in sample handling by identifying the optimal use of ACD anticoagulant, at ambient temperature (18-22°C) and up to 36 hours for storing blood samples best preserves monocyte function in mediating FcγR-dependent phagocytosis when compared to fresh. I also found that maintaining physiological pH throughout the MMA experimental procedure is crucial (Chapter 2). Next, I applied these conditions to clinical patient samples and still obtained relevant clinical correlations to IVIG-associated hemolytic responses (Chapter 3). Although the ideal situation is to process blood samples fresh, I conclude that the optimal conditions can be applied to clinical samples that cannot be processed immediately while maintaining cellular functions for later MMA evaluations.
4.3 Future Directions

The ultimate goal of this work is to assess and validate the predictive power of the MMA, and make this assay available for clinicians and hematologists when transfusing patients with a complex blood work history. Despite the long history of use and the availability of MMA in reference laboratories in the U.S., the majority of the published MMA data are retrospective in nature (Hunt et al, 1980; Schanfield et al, 1981; Branch et al, 1984). This raises the concern in regards to the enhanced detection of evanescent antibodies that were reactivated post-transfusion, which would have been below the detection level pre-transfusion and potentially result in delayed or even acute HTRs despite MMA crossmatch results (Williams et al, 2016). In addition, there is only one extensive publication that used MMA crossmatch results solely to select blood and transfuse alloimmunized patients without any complications (Noumsi et al, 2015), however, fresh allogeneic donors monocytes were used and the value of autologous monocytes cannot be assessed. In addition, the MMA is not a clinically recognized test and thus is not offered by accredited reference laboratories in Canada. Therefore, despite multiple attempts to crossmatch using the MMA results (Appendix Table A3), clinicians and patients may be reluctant to proceed with the transfusion of the identified units predicted by the MMA results to provide the most compatible transfusion. This is a quandary as prospective routine use of the MMA for selection of donor blood for transfusion would be necessary to prove its utility, which requires further investigations by using the MMA for crossmatching and transfusing alloimmunized patients, while correlating to clinical outcomes. This makes many clinicians reluctant to use the MMA in a prospective manner for donor blood selection.
Future optimization of additional storage variables should be examined, such as citrate phosphate dextrose adenine (CPDA) anticoagulant (Hess, 2006; Lu et al, 2011; Mincheff et al, 1993), or the potential of sample agitation and dilution to reduce granulocyte contamination during PBMC separation (Mallone et al, 2011). Despite these concerns, having reference laboratories, especially within the Canadian Blood Services, setting up and being able to perform MMA for transfusion clinicians in certain cases would be something that needs to be considered for the future of blood transfusion medicine.
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Appendices
Table A1. Reference ranges for standard laboratory blood tests.

<table>
<thead>
<tr>
<th>Laboratory Blood Tests</th>
<th>Reference Ranges*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/L)</td>
<td>Male: 140 – 180</td>
</tr>
<tr>
<td></td>
<td>Female: 120 – 160</td>
</tr>
<tr>
<td>White blood cells (10⁹/L)</td>
<td>4.0 – 11.0</td>
</tr>
<tr>
<td>Platelet (10⁹/L)</td>
<td>150 – 400</td>
</tr>
<tr>
<td>Reticulocyte count (10⁹/L)</td>
<td>30 – 110</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/L)</td>
<td>125 – 220</td>
</tr>
<tr>
<td>Haptoglobin (g/L)</td>
<td>0.3 – 2.0</td>
</tr>
<tr>
<td>Total/indirect bilirubin (μmol/L)</td>
<td>&lt; 23 / &lt; 18</td>
</tr>
<tr>
<td>Ferritin (μg/L)</td>
<td>Male: 22 – 275</td>
</tr>
<tr>
<td></td>
<td>Female: 4.6 – 204</td>
</tr>
<tr>
<td>C3 (g/L)</td>
<td>0.9 – 1.8</td>
</tr>
<tr>
<td>C4 (g/L)</td>
<td>0.1 – 0.4</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>&lt; 12</td>
</tr>
</tbody>
</table>

*From University Health Network
Table A2. *In vitro* IVIG opsonization of RBC conversions for the MMA assay.

<table>
<thead>
<tr>
<th>Weight (Kg)</th>
<th>Weight (lb)</th>
<th>Volume 10g/100mL IVIG (mL)</th>
<th><strong>Opsonization (v/v) of RBCs</strong></th>
<th><strong>Opsonization (v/v) of RBCs</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Men</em></td>
<td><em>Women</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dosage 1g/Kg</td>
<td>Dosage 2g/Kg</td>
<td>Dosage 1g/Kg</td>
</tr>
<tr>
<td>40.8</td>
<td>90</td>
<td>408.23</td>
<td>816.47</td>
<td>15</td>
</tr>
<tr>
<td>45.4</td>
<td>100</td>
<td>453.59</td>
<td>907.18</td>
<td>17</td>
</tr>
<tr>
<td>49.9</td>
<td>110</td>
<td>498.95</td>
<td>997.90</td>
<td>18</td>
</tr>
<tr>
<td>54.4</td>
<td>120</td>
<td>544.31</td>
<td>1088.62</td>
<td>19</td>
</tr>
<tr>
<td>59.0</td>
<td>130</td>
<td>589.67</td>
<td>1179.34</td>
<td>21</td>
</tr>
<tr>
<td>63.5</td>
<td>140</td>
<td>635.03</td>
<td>1270.06</td>
<td>22</td>
</tr>
<tr>
<td>68.0</td>
<td>150</td>
<td>680.39</td>
<td>1360.78</td>
<td>23</td>
</tr>
<tr>
<td>72.6</td>
<td>160</td>
<td>725.75</td>
<td>1451.50</td>
<td>24</td>
</tr>
<tr>
<td>77.1</td>
<td>170</td>
<td>771.11</td>
<td>1542.21</td>
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</tr>
<tr>
<td>81.6</td>
<td>180</td>
<td>816.47</td>
<td>1632.93</td>
<td>27</td>
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<td>86.2</td>
<td>190</td>
<td>861.83</td>
<td>1723.65</td>
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<tr>
<td>90.7</td>
<td>200</td>
<td>907.18</td>
<td>1814.37</td>
<td>29</td>
</tr>
<tr>
<td>95.3</td>
<td>210</td>
<td>952.54</td>
<td>1905.09</td>
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<td>99.8</td>
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<td>104.3</td>
<td>230</td>
<td>1043.26</td>
<td>2086.52</td>
<td>32</td>
</tr>
<tr>
<td>108.9</td>
<td>240</td>
<td>1088.62</td>
<td>2177.24</td>
<td>33</td>
</tr>
<tr>
<td>113.4</td>
<td>250</td>
<td>1133.98</td>
<td>2267.96</td>
<td>34</td>
</tr>
<tr>
<td>177.9</td>
<td>260</td>
<td>1179.34</td>
<td>2358.68</td>
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</tr>
<tr>
<td>122.5</td>
<td>270</td>
<td>1224.70</td>
<td>2449.40</td>
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</tr>
<tr>
<td>127.0</td>
<td>280</td>
<td>1270.06</td>
<td>2540.12</td>
<td>36</td>
</tr>
<tr>
<td>131.5</td>
<td>290</td>
<td>1315.42</td>
<td>2630.84</td>
<td>37</td>
</tr>
<tr>
<td>136.1</td>
<td>300</td>
<td>1360.78</td>
<td>2721.55</td>
<td>38</td>
</tr>
</tbody>
</table>

*Assumption that EVF constitutes 45% whole blood volume in men and 40% whole blood volume in women when packed.

**Assumption that BV is 5000mL across 90 to 300lb range.
### Table A3. Pre-transfusion MMA crossmatch results for alloimmunized patients using autologous PBMC.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cause(s)</th>
<th>Antibody identification</th>
<th>Date</th>
<th>Blood storage</th>
<th>Donor segments</th>
<th>IAT scores</th>
<th>MMA crossmatch (Mean ± SEM)</th>
<th>Transfusion outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Suspected low-grade macrophage activation syndrome; chronic hyperhemolysis; additional health conditions</td>
<td>Detected Anti-M</td>
<td>2015-03-05</td>
<td>Fresh in ACD</td>
<td>C0556-15-414016</td>
<td>N/A</td>
<td>Not significant* (0 ± 0)</td>
<td>Not transfused</td>
</tr>
<tr>
<td></td>
<td>Previously reported Anti-E, Anti-K and Anti-Fya</td>
<td></td>
<td></td>
<td></td>
<td>C0556-15-387355</td>
<td>N/A</td>
<td>Significant (5.09 ± 0.84)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Sickle cell disease patient previously developed high IgG titer post-RBC transfusion despite close to 100% matched RBC</td>
<td>N/A</td>
<td>2015-06-05</td>
<td>Fresh in ACD</td>
<td>C0556-15-406607</td>
<td>WR</td>
<td>Significant (9.93 ± 1.97)</td>
<td>Not transfused</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C0556-15-400942</td>
<td>1+</td>
<td>Not significant (4.13 ± 1.92)</td>
<td></td>
</tr>
<tr>
<td>3**</td>
<td>Patient with multiple alloantibodies; RBC units were identified and reserved for potential emergency transfusion during surgery</td>
<td>Anti-Yta</td>
<td>2015-08-23</td>
<td>Fresh in ACD</td>
<td>C0556-15-583005</td>
<td>2+</td>
<td>Not significant (1.69 ± 0.60)</td>
<td>Not transfused</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C0556-15-573380</td>
<td>2+</td>
<td>Not significant (0.88 ± 0.44)</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>C0556-15-573386</td>
<td>2+</td>
<td>Not significant (0.96 ± 0.78)</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>C0556-15-569481</td>
<td>3+</td>
<td>Not significant (0.62 ± 0.62)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C0521-15-531084</td>
<td>0</td>
<td>Not significant (1.05 ± 0.31)</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>C0556-15-569191</td>
<td>0</td>
<td>Not significant (1.10 ± 0.69)</td>
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<td></td>
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<td></td>
<td>C0556-15-535319</td>
<td>WR</td>
<td>Not significant (0.31 ± 0.31)</td>
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</tr>
</tbody>
</table>

*MMA crossmatch significance as determined by the previously published cut-off of <5% phagocytosed or attached RBCs per 100 monocytes (Arndt & Garraty, 2004; Noumsi et al, 2015).

**Published in Tong et al, 2016b.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Cause(s)</th>
<th>Antibody identification</th>
<th>Date</th>
<th>Blood storage</th>
<th>Donor segments</th>
<th>IAT scores</th>
<th>MMA crossmatch (Mean ± SEM)</th>
<th>Transfusion outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Sickle cell disease and emergency surgery; RBC units were identified and reserved for potential post-surgery follow-up transfusion</td>
<td>Potential anti-Fy5 and/or anti-Fy3</td>
<td>2015-09-02</td>
<td>Fresh in ACD</td>
<td>C0556-15-590780</td>
<td>0</td>
<td>Not significant (0.15 ± 0.15)</td>
<td>Not transfused</td>
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<td></td>
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<td></td>
<td>C0556-15-592468</td>
<td>0</td>
<td>Not significant (0.16 ± 0.16)</td>
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<td></td>
<td></td>
<td>C0556-15-594777</td>
<td>WR</td>
<td>Not significant (0 ± 0)</td>
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<td></td>
<td>C0556-15-579436</td>
<td>WR</td>
<td>Not significant (0.47 ± 0.26)</td>
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<td>C0556-15-497586</td>
<td>1+</td>
<td>Not significant (0 ± 0)</td>
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<td>C0556-15-583379</td>
<td>1+</td>
<td>Not significant (0.16 ± 0.16)</td>
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<td>C0556-15-574620</td>
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<td>Not significant (0 ± 0)</td>
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<td>C0556-15-588218</td>
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<td>Not significant (0 ± 0)</td>
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<td>C0556-15-587661</td>
<td>2+</td>
<td>Not significant (0.28 ± 0.16)</td>
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<td>C0556-15-590372</td>
<td>2+</td>
<td>Not significant (0.59 ± 0.38)</td>
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<td>2015-10-05</td>
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<td>C0556-15-584229</td>
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<td>Not significant (0 ± 0)</td>
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<td>C0556-15-606727</td>
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<td>Not significant (0.13 ± 0.13)</td>
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<td>2015-10-13</td>
<td>Fresh in ACD</td>
<td>C0556-15-584229</td>
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<td>Not transfused</td>
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<td>C0556-15-606727</td>
<td>0</td>
<td>Not significant (1.11 ± 0.26)</td>
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