Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Justine S. Liepkalns Date

Clearance and Resistance of Red Blood Cells during Incompatible Transfusions

By

Justine S. Liepkalns
B.S., Eckerd College, 2004
M.S., Tufts University, 2006

Advisor: James C. Zimring, M.D., Ph.D.

An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Immunology and Molecular Pathogenesis

2012

Abstract

Clearance and Resistance of Red Blood Cells during Incompatible Transfusions

By Justine S. Liepkalns

Transfusion therapy is widely used to treat acute and chronic anemia. Although compatibility testing has been used to avoid pathophysiologies such as hemolytic transfusion reactions (HTRs), incompatible transfusions still occur. Some incompatible transfusions do not lead to pathophysiology and the survival of incompatible RBCs is normal. This dissertation sought to study RBC survival in the context of a novel murine model of incompatible transfusions.

Studies herein were done using monoclonal antibodies against the human blood group glycophorin A (hGPA) and Duffy (as part of a fusion protein called HOD) antigens. Anti-Duffy antibodies were found to clear HOD expressing RBCs via Fc-receptors. In contrast, anti-hGPA antibodies were found to clear RBCs via a third novel biphasic mechanism. In the first phase, anti-hGPA antibodies agglutinate RBCs, sequestering them from circulation. During the second phase, phagocytic cells are required for removal of the sequestered RBCs independent of Fc-receptors and of complement. A coinciding cytokine burst was found to require Fc-receptors, which suggests a decoupling of phagocytosis and cytokine secretion during the clearance of incompatible hGPA RBCs.

With the knowledge of the clearance pathways of RBCs bearing these 2 antigens, we investigated the RBC's ability to survive *in vivo*. Not all hGPA and HOD RBCs cleared when faced with a bolus of anti-hGPA and anti-Duffy antibodies, respectively. During hGPA or HOD incompatible transfusions, a population of RBCs was found to be

resistant. Resistance of hGPA or HOD RBCs was found to not require C3, contradicting previous findings. A titration of anti-hGPA antibody-mediated clearance suggests a spectrum of RBC susceptibly among hGPA RBCs. Incompatible transfusion studies with HOD RBCs suggest that the resulting resistant RBCs did not acquire the ability to resist but rather resistance is an innate quality of that population of RBCs.

Overall, incompatible RBC clearance pathways seem to vary among blood group antigens and/or binding antibody. The resulting RBC resistance may be relative to the blood group in question. This thesis elucidates a novel RBC clearance pathway and confirms incompatible RBC resistance as a phenomenon. In this particular system, resistance was not found to require Complement as previously suggested in other systems.

Clearance and Resistance of Red Blood Cells during Incompatible Transfusions

By

Justine S. Liepkalns
B.S., Eckerd College, 2004
M.S., Tufts University, 2006

Advisor: James C. Zimring, M.D., Ph.D.

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Immunology and Molecular Pathogenesis

2012

Acknowledgements

I would like to thank Dr. James Zimring for being the best mentor I could have ever asked for in addition to being a wonderful friend. Joining your lab was the best decision of my graduate career. Your scientific guidance has not only shaped the way I teach but the way I think. I can't turn it off now, I'm always thinking of what could be the alternate interpretation of the data. I will truly miss working with you and our long walks in Lullwater Park and along the Seine.

I am grateful to my committee members for their helpful comments and invaluable expert advice throughout my Ph.D. training.

I am indebted to many people for advice, support and assistance, in particular the members of the Zimring lab (past and present) and Dr. Jeanne Hendrickson. Everyone was always willing to lend a helping hand. Thank you for making the environment in the lab conducive to scientific discussions and just fun to work in. Max Desmarets, thanks for holding the French Quarter with me (Kathryn Girard, thanks for making it the Italian/French Quarter after that). Seema Patel, I'm glad I followed you around the lab rotations. Shea Cadwell, thank you for showing me the ropes when I first started. Nikki Smith, thank you for always making everyone's birthday special.

I have made wonderful friends prior to coming to Emory and during the course of my education here. You all know who you are. Nicole Huebener, you have been with me through it all, ever since Knox 14. You have shown me what true friendship really means. Mandy Ellenburg, we have created our own language over the years. Kelly Gillen, even though we don't live in Boston anymore, I always think of the good times we had when I go back. Priya Sundararajan, you always push me to be more and more adventurous (I'm looking forward to our next one with Ramya). Danielle Dean, you have made Emory a cozy home for me by instantly welcoming me to the area and by being the lovely person that you are. Lisa 'Mclay' Schelde, we have been friends since our

interview weekend (as soon as we stepped off the plane). Emory was even reluctant to let us leave. Noah Alberts-Grill, you have also been one of my first friends at Emory. I find myself constantly learning from you. Mike Elrod, you have brought to Atlanta what I missed from Eckerd. The monthly get-togethers with the boys (Mike, Noah, Chuck, Richard) are a major source of belly laughs. I look forward to our next road trip together, in particular the one in Dean Winchester's Impala. Chris Gilson, I always enjoyed our weekly lunches. Kathy Reding, our Tough Mudder and Warrior Dash runs have made goat herders around the world jealous. Katherine Bryant, thank you for always being supportive and protective of the ones you care about. Also, to my basketball buddies, Tony Lau, Dana Tucker, David Brown and Konrad Bradley, thanks for playing one of my favorite sports with me. Thank you Diamond for being a good friend. Thank you to the rest of my Emory friends, who have made Emory my home for the past few years (Katie Carroll, Ryan Powers, Ivana Ferrer, Ashley Bennett, Terrell Brotherton, Shawn Alter, Cathy Gavile, Brian Gaudette, Matt Brentnall, Cecilia Prudente, Chase Bourke, Vickie Jeisy-Scott, Mimi & Nico and many many more). Thank you to my ORDER crew for inspiring me to be creative in the classroom and to the SIRE crew for educating me on how to be a good mentor. Thank you Cari Fritz-French for helping me while I ran my first lecture course (Bio120). Thank you to my past labs, including Dr. Mitch McVey and the fly pushers. Thank you to my friends from Eckerd for bringing back the good ol'days (Emmanuel Nkrumah, Steve Bordeleau, Lindsey Phelps, Jennifer Ortiz, Maddy Franco-Ortiz, *et al.*). Thank you Andrew Smith for being one of my first friends when I first moved to the States. Thank you to my boxing chicks. Tricia, mon amie, you and I have gone through it together. Thank you to my past and current roommates for being my only human contact during those long lab weeks. Katherine Bryant and Dan Coppeto, I'm looking forward to another great year together. Amanda Rogers, I have learned so much from you. I know you will continue to inspire much needed change in the world.

Thank you all for being there to support me, to have fun with me and for being all around fabulous.

Most of all, I would like to thank my family, in particular my parents and brother. You have made my life so much more wonderful. I love you more than words can say, although I'm going to try. Thank you to my mother who will always pick up the phone to talk to me no matter the time of day (or night), just to calm me down and to come up with ways to fix the situation. Thank you for worrying about me and for being my best girlfriend. Je te dis tout ce que j'ai sur le coeur et ça me soulage, merci Maman. Thank you to my brother, Jimmy, for being my best friend. We always know when the other one is going to break into song ('so many more to see'). Je t'aime de tout mon coeur. To my father, Daddy not only did you help me fall asleep when I was young by bringing me warm milk and honey, but you still know what to say now to make me feel better.

Table of contents

List of Figures

Chapter 1

Introduction

Historical background

Throughout history, blood has sparked human interest. From 770 BC to 221 BC, the Chinese Huang Di Nei Ching believed that blood contained the soul of the person. In 450 BC, Hippocrates believed that disease was caused by an imbalance of the 4 humors: black bile, yellow bile, phlegm and blood. For centuries, throughout much of Europe, blood was generally considered to be a tonic for various ailments. Romans drank blood from departed gladiators in the hopes of gaining their vigor.[1]

This idea of blood being a rejuvenating agent transitioned into the medical field. In 1492, Pope Innocent VIII fell into a coma and was given blood orally from 3 young boys who were promised a ducat (a French coin at the time). Unfortunately, neither the boys not the pope survived the procedure. Later, in 1667, Jean Baptiste Denis (one of Louis XIV's physicians) performed multiple transfusions on a 34-year-old patient afflicted with mental illness named Antoine Mauroy who was known for beating his wife, running through the streets after stripping off his clothes and setting houses on fire. Denis thought that blood from animals was purer than human's blood, which was tainted by man's "debauchery and irregularities in eating and drinking." Denis, therefore, transfused calves' blood in the hopes of replacing the 'bad' human blood with 'good' animal blood. Mauroy complained of heat moving through his arm during the 1st transfusion. During a 2nd transfusion, more blood was used and the patient complained of kidney pains. Mauroy later vomited and excreted urine "black as soot." Physicians thought it to be the 'bad spirit' leaving the patient's body. It was later understood by the medical community that Mauroy's black urine contained, instead, dead red blood cells (RBCs) from the transfused animal's blood. Mauroy eventually died shortly after the series of transfusions. This matter went to court for malpractice, however, once an autopsy was performed, Mauroy was found to have been poisoned with arsenic (by his wife). Even though Denis was exonerated, human transfusions were banned by the French and English courts as well as by much of Europe.[1]

It was not until the late 18th century, in 1795, that the first human-to-human transfusion was performed. Jean Henry Leacock's dissertation in 1816 on "the transfusion of blood in extreme cases of hemorrhage" and his subsequent animal research demonstrated the importance of matching donor and recipient of any transfusion by species. Later, in August 1825, James Blundell performed the first recorded successful human-to-human transfusion to treat postpartum hemorrhaging in a woman using her husband's blood. After a few more successful transfusions in Europe and the use of this therapy during the American Civil War by the Union Army, Emil Ponfick and Leonard Landois further investigated the issue of incompatibility between species during the late 1800s. It was with animal models that Ponfick described the symptoms of incompatible transfusions, including hemorrhage and "congestion" of the kidneys, lungs and liver. Landois' experiment of mixing human blood and serum from other species *ex vivo* (known as compatibility testing) revealed that the blood was incompatible due to **hemolysis** (RBC lysis) *in vitro*. It was not until the discovery of ABO in 1901, that all human blood was no longer thought to be the same. Karl Landsteiner was awarded the Nobel Prize in Physiology or Medicine in 1930 "for his discovery of human blood groups." Richard Weil was the first to perform blood typing (testing the blood for presence of specific blood group antigens) and testing for compatibility (cross-matching donor and recipient) a few years later. A study by Ottenberg and Kaliski, later reinforced the importance of cross-matching with the transfusion of 128 patients.[2] During **cross-matching**, RBCs from the donor are mixed with serum from the recipient. If this mixture induces hemolysis or agglutination, the recipient is considered incompatible for the blood tested due to presence of antibodies against donor RBC antigens.

Blood group discoveries

Since the discovery of ABO, many other blood group antigens have been revealed on the human RBC. This includes over 200 different blood group antigens (assigned to 29 blood group systems).[3,4] After ABO, P and MN (now known as MNS as the S antigen was added later on) were discovered in 1927. In the 1930s, Landsteiner-Weiner was discovered with the injection of Rhesus blood into rabbits. Almost a decade later, Rh was revealed in mothers of stillborns (1939) and transfused patients (1940).[5]

The nomenclature for these blood groups varies. Some have been named after the person in whom it was discovered (the carrier or the reacting recipient), some after the scientist who described it, and still others were named alphabetically or numerically.[3] Currently, the nomenclature used is in accordance with the international society of blood transfusion (ISBT) working party. Dr. B.P.L. Moore initiated this organization in order to come up with a naming system for blood group antigens that can easily be read by people and understood by computers while still referencing the gene of the antigen.[6]

Most blood group antigens are carried by membrane proteins having a single-pass (type I and II), multi-pass, or glycosylphosphatidylinositol (GPI)-linked structure.[3,7] Others, however, do not fall into these categories, such as blood groups Lewis and Chido-Rodgers, which are acquired from the host plasma. ABO, H and P blood group systems are carbohydrates attached to surface proteins or lipids.[7]

Clinical significance of recipient antibodies

Due to the variety of blood groups found on the human RBC, cross-matching has become an essential part of transfusion medicine. Differences that exist on the surface of RBCs are called alloantigens to which alloantibodies can bind and elicit a reaction from the host. Exposure to RBC alloantigens occurs through transfusions or pregnancy potentially resulting in a **hemolytic transfusion reaction (HTR)** or **hemolytic disease of the fetus**

and newborn (HDFN), respectively. The level of reaction induced by binding antibodies is referred to as **clinical significance**. Antibodies against A and B of the ABO system and Rhesus are among the most clinically significant and are a common concern in patients undergoing a transfusion or carrying a child. In addition to these antibodies, those against Diego, Duffy, Kell, Kidd, Vel blood groups and S, s and U antigens of the MNS system are also usually clinically significant. On the other hand, antibodies against Lewis (Le^b), Cost, Knops, Chido/Rogers, JMH, Xg(a) are generally not clinically significant. A few blood group antigens only sometimes lead to adverse reactions such as Colton, Cromer, Dombrock, Gerbich, Indian, Landsteiner-Weiner, Scianna. Other blood groups are only clinically significant if bound by antibody at 37^{o}C, among those are Lutheran, Lewis (Le^a), A_1 of the ABO system and M, N of the MNS system.[3] Cases have been reported of patients undergoing no reaction upon ABO incompatible transfusion.[8] It has been suggested that only about half of ABO incompatible transfusions lead to an HTR. The frequency of these reactions seems to vary according to blood group antigens, the reason for which is unknown. It is also unclear why some patients have no response while others suffer severe, potentially fatal, outcomes with transfusions of the same incompatible blood group.

Direct antiglobulin tests (DAT) (or Coombs' tests)[9] are used to determine antibody binding by exposing collected RBCs to secondary antibodies (typically rabbit anti-human Immunoglobulins (Igs)) specific for the Fc portion of antibodies. This test is useful in quantifying antibodies on the surface of RBCs by visualizing the level of agglutination. In studies performed in this thesis, DATs of RBCs were performed using a fluorescent secondary antibody and flow cytometry to quantify RBC surface fluorescence on a per cell basis.

The ABO blood group system

ABO's discovery in 1901 contradicted the general belief that all human blood was the same.[1] ABO blood type frequencies vary according to different human populations. This variability is due to a glycosyltransferase expression, an enzyme responsible for sequentially adding certain monosaccharides in specific linkages leading to a precursor chain, called the H antigen. If an individual has the glycosyltransferase for the A or B phenotype, most of the H antigens are converted to the corresponding antigen.[1] The glycosyltransferases for the A and B antigens differ by 4 amino acids (of 354 amino acids), while the O allele has only a single nucleotide deletion resulting in a frame shift and a protein with no enzymatic activity.[10] Other mutations result in subgroup phenotypes within A, B, and O. There have so far been 41 different A, 18 B, and 61 O subgroup alleles observed in humans.[11] These subgroups vary in structure and level of surface expression on red cells. The A antigen has 2 major subgroups, known as A_1 and A_2, which have structural differences significant enough for possible alloantibody formation in recipients. About 80% of group A phenotypes are A_1 and express more antigens per red cell than A_2 carrying individuals. The frequency of ABO antigens are as follows: $O>>A_2>B>A_2B>A_1>A_1B$. Another rare phenotype within the ABO blood group is the Bombay phenotype, which does not even express the H antigen. These individuals need to be transfused with blood that lacks the H antigen all together (i.e. they can only receive transfusions from other Bombay donors due to anti-H antibodies).[11] In addition to assigning blood types within the ABO system there is also the distinguishing nomenclature of *Se* (secretor) and *se* (non-secretor) as some individuals were found to have these blood group sugars in their secretions.[12]

Even though the ABO blood group system is the most well known, it is also fairly unique. Due to the nature of this blood group, individuals who lack the A and B antigens develop IgM antibodies against A and B (respectively) soon after birth without exposure to these antigens via transfusion. The production of these antibodies (dubbed "natural

antibodies") peaks at 5 to 10 years of age and is in response to enteric bacteria (*Escherichia coli*), which were found to also express these sugars.[11,13,14]

The 'natural' production of these antibodies poses a particular problem in transfusion medicine because these antibodies are commonly found in the population as no prior exposure to foreign ABO blood group antigen is necessaryfor their production. In addition, upon transfusion, these Anti-A and anti-B antibodies can elicit a potentially fatal HTR and are considered to be some of the most clinically significant antibodies.[5]

The MNS blood group system

The MN antigens were the second blood group antigens to be discovered, later joined by S.[5] This system includes 2 different proteins known as **glycophorin A (GPA)** and glycophorin B (GPB). This highly polymorphic system has 40 different antigens ranging from mild to severe reactions upon antibody binding. Glycophorin E is also part of the gene family although it is not clear whether it is expressed on RBCs. GPA and GPB are type I single-pass membrane sialoglycoproteins (with the N-terminus eposed extracellularly). GPB has 11 possible O-glycan sites and no N-glycan site, with approximately a molecular weight of about 25,000 Da. There are about 200,000 GPB copies per RBC. S and s are antithetical antigens found on GPB at position 29. S has a lower occurrence (55% Whites, 30.5% Blacks) than s (89% Whites, 94% Blacks). The S−s− phenotype is rare and mostly found in people of African descent, due to a deletion or alteration of GPB. This is also the case in individuals said to be negative for the U antigen (Valine at position 33 and Proline 39) also found on GPB.[1,3]

The GPA antigen is expressed at approximately 800,000 to 1,000,000 copies per RBC. GPA is 43,000 Da and has about 15 O-glycan sites and one N-glycan site. The M and N antigens are a result of an amino acid change at positions 1 and 5. The M version of the antigen has a Serine at position 1, a Glycine at position 5 and is rarely implicated in HTR

and hemolytic disease of the newborn (HDN). N GPA has a Leucine at position 1 and Glutamic Acid at position 5, which is also the case with GPB. The N variant of GPA elicits antibodies that lead to no noticeable pathophysiology and are therefore considered not clinically significant. Rare S-s-U individuals can make anti-U (against GPB), which can cross react with N GPA. These antibodies are considered to be clinically significant. Both M and N antigens have fairly high frequencies (around 75% in both Blacks and Whites). The human RBC will either carry M+N−, M−N+ or M+N+ antigens.[1,3]

Due to the high levels of sialic acid on GPA and GPB, a layer of negative charge is generated which is thought to be responsible for preventing RBCs from adhering to each other and to the vessel walls. A rare phenotype with RBCs lacking the GPA protein (called En(a−)), however do not suffer disease. Unfortunately, GPA can also function as a receptor for *Plasmodium falciparum* (the causal agent of Malaria), which does not have the capacity to bind RBCs with the En(a-) phenotype.[15] An additional possible GPA function is preventing the formation of C5b-7 of the complement system.[16]

GPA can also form homo- and hetero-dimers because of a cluster of positively charged amino acids well placed for interactions with negatively charged proteins. Interactions with protein 2.1 (Ankyrin), 4.1, 4.9, 6, and Band 3 (carries Diego blood group antigens) have been observed.[17-19] These proteins are known to interact with the RBC cytoskeleton. Specifically, Ankyrin and protein 4.1 mediate the association between membrane and RBC cytoskeleton.[20] Moreover, Ankyrin links Band 3 to spectrin, a protofilament stabilized by proteins such as protein 4.9 and may be responsible for the RBC's general flexibility.[20,21] Protein 4.1 enhances spectrin affinity to actin by binding 2 spectrin subunits.[20] Other associations with GPA involve chaperoning integral membrane proteins to the plasma membrane.[3,4,22,23]

Investigations on membrane flexibility, using an ektacytometer, have shown that binding of GPA by lectin or antibody increases rigidity of the RBC membrane.[24-27] It has

been proposed that this increased rigidity induced by GPA binding could be a mechanism of clearing infected RBCs from the circulation (such as with Malaria).[4]

Experimentally, the glycophorin protein can be a useful tool. Since glycophorins are frequently present on RBCs, it is used by investigators as an RBC marker.[28] In a murine model, a monoclonal antibody called TER119, used to identify murine RBCs, binds to a GPA-associated epitope and does not cross react with the human form of GPA.[18,29] A transgenic mouse expressing the human form of glycophorin A has since been created in order to study the protein *in vivo*. The human protein was shown to not dimerize with the murine glycophorin A but may compete for Band 3 binding.[18]

The Duffy blood group system

In 1950, after receiving multiple transfusions, a male patient with Hemophilia A produced antibodies against a blood antigen later named Duffy (after the patient).[30] The Duffy multipass membrane glycoprotein resembles the G-protein coupled receptor with an extracellular N-terminus. The clinical significance of anti-Duffy antibodies ranges from mild to severe.[3,5,31] The Duffy group system has 6 different antigens including Fy^a, Fy^b, Fy^3 and Fy^6. Fy^a and Fy^b are antithetical antigens of each other with a difference in only one amino acid at position 44 (glycine and aspartic acid, respectively). The frequency of Fy^a is high among Asian and Thai populations (over 95%), moderate in Whites (66%) and is fairly low in Black populations (10%). Fy^b frequency is higher in Whites (83%), however is low in Thai (31%), Black (23%), Japanese (18.5%), and Chinese (9.2%) populations. Mild to severe clinical significant reactions can result from antibodies against Fy^a and Fy^b, with anti-Fy^b rarely inducing mild forms of HDN. F(a−b−) phenotype also exists (predominantly in people of African descent). This phenotype is due to a disruptive amino acid substitution resulting in a Duffy null phenotype on RBCs, however Duffy is still expressed normally on non-erythroid cells. Fy^6 (Serine-31 and aspartic acid-40) has only been defined in a murine model using

monoclonal antibodies. The Fy^3 antigen is found on the 3rd extra cellular loop of the Duffy antigen. When individuals are missing the Fy^3 antigen, the Duffy protein is not expressed on their RBCs. The frequency of the Fy^3 blood group is found in nearly 100% in White, Oriental and Yemen Jewish populations, however it is only found in 32% of Blacks.[3] The Duffy protein was identified as a receptor for *Plasmodium vivax* potentially explaining the lack of Duffy expression in persons originating from West Africa.[32] An additional function that Duffy may have is receptor for chemokines (cytokines that induce chemotaxis). It has been hypothesized that Duffy acts as a sink to dampen excess levels of chemokines released into the circulation.[1] Duffy has been shown to bind members of the C-X-C chemokine family, such as interleukin-8 (IL-8) and melanoma growth stimulatory activity (MGSA), as well as members of the C-C family including monocyte chemotactic peptide-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1) and RANTES (regulated upon activation, normally T-cell expressed and secreted).[33,34]

Blood transfusion therapy

Transfusions were not routinely used until 25 years after World War II,[5] during which direct transfusions from donor artery to recipient vein were phased out and transfusions of stored blood were increasingly practiced.[5,11,35] Loutit and Mollison in 1943 had developed acid citrate dextrose (ACD) solution for blood collection in order to prevent clotting of donated blood.[11] As a result, it was possible to store blood for weeks instead of days. Prior to the use of ACD, sodium citrate was used at a small scale during World War I as an anti-coagulant, however, the blood could only be stored for a few days.

Transfusion Therapy and Anemia

Transfusions are performed in order to treat anemia (acute or chronic) and coagulation disorders. Anemia is defined as having lower than normal levels of hemoglobin

(Hb) per blood volume. Normal Hb levels in men are from 138 to 172g/L, and for women from 120 to 156 g/L,[36] however these ranges vary according to the source. Hematocrit (Hct) levels are also used to determine total RBC content in the blood. Normal levels in men are between 41-50% and women between 35-46%.[36] After considerable blood loss, the body reacts by redistributing blood flow via selective vasoconstriction towards the brain and the heart, away from the skin and subcutaneous tissues resulting in skin pallor or jaundice.[37] Cardiac output is also increased as a compensatory measure. A study on pigs by Räsänen showed that a 70% decrease in blood Hb level resulted in a sustained 22% elevation in heart rate and an initial gradual 44% elevated cardiac output.[38] A human study showed similar trends with a decrease in Hb levels from normal (125-134g/L) to 45-54g/L, during which a 59% increase (58 to 92 beats per min) in heart rate occurred.[39] Signs and symptoms of anemia include faintness, dizziness, thirst, sweating, weak / rapid pulse, and rapid respiration. In severe anemia with Hb levels below 70g/L can be associated with headache, vertigo, seeing spots, and bizarre behaviors.[36]

The body can tolerate some level of anemia although this depends on the overall blood loss, the amount lost overtime, and the rapidity of the blood loss. A sudden loss of 1/3 of a patient's blood volume can be fatal, whereas a gradual loss of 2/3 of blood volume over the course of 24 hours can be tolerated.[36] Massive blood loss can be managed with large amounts of volume expanders (such as saline or crystalloid solutions) to avoid hypovelimia and shock, followed by a red cell transfusion.[37]

Patients that need a transfusion may be suffering from various other ailments, which can affect their ability to tolerate anemia. A study on anemic baboons showed that hemoglobin levels as low as 50 to 30g/L can be tolerated with resulting isochemic electrocardiographic changes and depressed ventricular function in otherwise healthy baboons.[1,40] However, in animals with coronary stenosis, these heart complications are noticed at 70 to 100g/L of Hb.[1]

Even though some anemia can be tolerated, human observations in patients refusing blood transfusions due to religious beliefs and in areas where transfusions are not available demonstrated a higher likelihood of morbidity and mortality in patients with low Hb or hematocrits, highlighting the importance of transfusion therapy.[41-45] A series of elective surgeries were performed on patients refusing blood transfusions with blood loss ranging from less than 500ml to more than 2L during surgery. Some patients died after losing more than 500ml of blood (9/43 and 4/54 patients),[42,43] which was exacerbated with low preoperative Hb levels.[42,43,45] Other observations in postpartum hemorrhaging women demonstrated that as hematocrit levels decreased, mortality increased with a total of 18 out of 92 deaths.[41] In 542 Jehovah's Witness patients undergoing various surgeries (including cardiac surgery), 15 out of the 51 deaths were interpreted as being linked to excessive blood loss.[46] Age can also play a role in tolerance of lower than normal hemoglobin levels.[1] These studies show that patients can endure low Hb levels to a certain extent, however age and other medical conditions may make the patient more sensitive to blood loss, reinforcing the importance of transfusions.

Clinically, each unit of packed red blood cell used to treat anemia contains about 200ml of RBCs at a hematocrit of 70-80% in plasma (about 50-75ml of plasma).[47] Each unit of blood is given in the course of 4 hours. If given any slower, the patient runs the risk of getting a bacterial infection.[36] Physicians determine the cause of anemia by looking at the patient's history and their symptoms as well as their RBC's physical characteristics such as morphology and size. The mean cell volume (MCV), for which the normal range is between 80 and 100fL, is an indication of the type of anemia. Microcytic anemias (MCV of <80 fL) can be associated with low iron or reduced heme synthesis. Macrocytic anemias (MCV of >100 fL) can be due to liver disease, alcoholism, folate deficiency, deficiency in vitamin B_{12},[48] and to stress on erythropoiesis, which is due to the rapid development of new RBCs in response to acute anemic stress.[49] Finally, normacytic anemias (MCV between 80 and

100fL) can be a result of inflammation (from chronic disease), growth factor deficiencies (thyroid hormone, testosterone), or from pathologies that disrupt normal bone marrow architecture.[50]

Chronic Transfusions

Conditions leading to chronic anemia include thalassemia,[51] sickle cell anemia,[52] autoimmune hemolytic anemia (AIHA),[53] leukemia,[54] other cancers, and conditions of chronic low volume blood loss such as colonic polyps. Iron-overload is a common problem for patients needing multiple transfusions and will be discussed later in this chapter. Iron is important for RBC function in oxygen binding and release. It is typically contained within the hemoglobin complex in the RBC, however upon RBC death the free iron can lead to pathophysiology. Patients requiring transfusions experience high levels of RBC death and therefore are at risk of enduring iron overload.

Blood transfusions are aimed to maintain a Hb level of 90-100g/L[51] in order to increase oxygen-carrying capacity by correcting anemia as well as suppressing erythropoiesis and perhaps inhibiting increases in iron gastrointestinal absorption.[55] If transfusion is avoided, iron accumulation occurs as patients absorb more iron than they need,[55,56] however this is a double-edged sword since transfusions also lead to iron accumulation. Since the spleen is a major organ for RBC removal (discussed later in this chapter), splenectomies can help decrease transfusion requirements and may be needed for patients requiring transfusions exceeding 200ml packed RBCs per kg body weight.[57]

Halt in erythropoiesis (RBC production) leads to a 10% per week (or 1% per day) RBC decline in an individual. Hemolysis or chronic blood loss may be involved if a greater rate of decline is observed.[36] In human adult males, the average RBC volume is 30ml/kg with an average lifespan of 115 days. RBC daily production is on average 30ml of RBCs/kg per day. A 70kg man produces approximately 18ml of RBCs per day, 12ml if only 100g/L Hb

is needed to be maintained. In order to maintain the same Hb levels in a patient with total RBC production failure, the volume of blood to be transfused per day is 24ml. This volume is doubled, however, since RBCs collected from donors and stored are of mixed ages with an average lifespan of 57.7days. Taking into consideration some loss of blood from storage conditions and RBCs left in the collection bag, 200ml (1 unit) of RBCs per week are needed to maintain a minimum Hb level of 100g/L with transfusion.[5]

Unfortunately, transfusion therapy can be limited by the alloimmunization that sometimes ensues. Various studies have investigated the level of alloimmunization (and autoimmunization) that results from the frequent use of transfusion therapy and have found the rate of alloimmunization to range from 0.2% to 22.6% (and of autoimmunization from 0.1% to 1.7%).[58-65] These studies were done in different countries and with different numbers of total participants. Among the larger studies, Spanos *et al*. found that 220 out of 973 (22.6%) Greeks were alloimmunized[60], which was supported by another study from Michail-Merianou *et al*. who found a similar rate of alloimmunization (23 out of 120 or 19.2%)[59] in Greek participants. Among the 220 alloimmunized participants in the Spanos study, 160 (72%) were immunized against Rhesus, 140 (64%) against Kell, 37 (16%) against MNS, and 19 against Duffy (8%).[60] About half of alloimmunized patients had antibodies against more than one blood group (30% had antibodies against more than 3). An additional finding in this study was that if patients started the transfusion regimen prior to three years of age, 20.9% (196/937) developed alloantibodies while if the regimen started after the age of three, 47.8% (48/101) were found to have alloantibodies.[60] A Canadian study summarized 1/36 (2.8%) transfused patients (with a total of 2082 follow ups) were alloimmunized and added that other studies found frequencies of 1/128 (0.8%) and 1/461 (0.2%).[61]

Studies done in Iran found 12.1% (101/835)[64] and 5.3% (38/711)[63] alloimmunization rates. Among the 101 immunized patients, 22% (22/101) had antibodies against more than

one blood group.[64] A cooperative study done in Italy with 1,435 patient participation found a 5.2% alloimmunization rate with clinically significant antibodies. Alloantibodies included those against blood groups RhD (35.3%), Kell (33.1%), Duffy (6.6%), and MNS (3.7%).[58] The rate of alloimmunizations is relatively low when compared to immune responses to disease during which antibody responses are close to 100%. The low rate of alloimmunization is not likely due to a weak immunogenic nature of the RBC alloantigen because solid organ transplants lead to near 100% rejection without potent pharmacological intervention.[66]

The majority of these studies were performed in young patients. The rate of alloimmunization may be increased with the increased number of transfusions in adults, as one study found to be the case. [60] After cross-matching blood for ABO, these studies showed that patients become alloimmunized against a variety of other blood groups including RhD, Kell, Duffy, and MNS. This variety in alloantibodies found in serums of chronically transfused patients makes it very difficult to find compatible units. Not only is it not guaranteed that a compatible unit will be found but this search can be very time consuming (may take up to 24-48 hours) and delay a much needed transfusion.[1] The problem of alloimmunization persists essentially unaltered today. Transfusion is a life-sustaining therapy for patients with chronic anemia. As demonstrated by the above studies, alloimmunization increases with transfusion frequency, therefore decreasing the number of available compatible units for these patients. If no compatible blood is found, patients unfortunately succumb to the disease.[66]

Despite the risks involved in incompatible transfusions, physicians at times choose the 'least incompatible' units and transfuse the smallest volume needed to improve life-threatening conditions while closely monitoring the patient.[1,67] Transfusion of incompatible units may also proceed if blood bank evaluations are not completed in time. When cardiac or cerebral functions are compromised, patients are transfused regardless of compatibility.[1]

Conditions causing chronic anemia

Patients with certain forms of **thalassemia** require transfusions as early as 6 months of age in order to survive. Hb levels in these patients range from 20 to 70g/L and levels as low as 50g/L are well tolerated.[5] Hb is a tetramer with 2 α-globin and 2 β-globin subunits, mutations of which lead to α- and β-thalassemias, respectively. α-thalassemia is usually associated with only mild anemia. β-Thalassemia has 3 different phenotypes: minor, intermediate, and major (Cooley's anemia) with 200 different mutations leading to reduced or abolished expression of β-globin genes.[68] Patients with Cooley's form have severe anemia (less than 80g/L Hb)[68] and frequently require transfusions while those with intermediate thalassemia require no or occasional transfusions. Symptoms include bone deformities, jaundice, and leg ulcers.[51] Patients undergoing hyper-transfusions early in life were found to maintain a Hb level of about 92g/L pre-transfusion and about 130g/L post-transfusion with no observable change in bone development.[69] If left untreated children with Cooley's anemia die by the age 5, however with occasional transfusions patients live into their second decade.[51] Once iron chelators were added to their treatment regimen, patients lived well into adulthood.[70] Despite some risks involved in transfusions, it is still a life sustaining therapy for patients with Cooley's anemia; thus, avoiding transfusion is not a viable strategy.

Another hemoglobinopathy is the well-known **sickle cell anemia (or sickle cell disease (SCD))**. First described in 1910,[71] SCD is seen in 2-3% of African-Americans[36] with a rate of approximately 1 in 350 African-Americans born with homozygous HbSS.[72] Various phenotypes exist including HbSS (homozygous for the Sickle-cell gene), HbS/C (heterozygous), or HbS/b (with a form of thalassemia).[71] As opposed to Thalassemia, the Hb in SCD is present in normal amounts and alpha/beta ratios; however the Hb is misshapen resulting in a hydrophobic interaction between Hb molecules forming large Hb polymers in

deoxygenated RBCs not seen in normal RBCs.[73] The resulting banana, or sickle shaped, RBCs causes anemia due to hemolysis and shortened RBC lifespan, as well as vasculopathy characterized by increased vaso-occlusion and activation of coagulation cascade. The major causes of morbidity and mortality are infection, stroke, splenic sequestration, pulmonary thromboemboli, renal failure, and pulmonary hypertension (in decreasing order of frequency). Transfusion therapy is used to not only increase oxygen carrying capacity of RBCs but also to decrease the viscosity and improve blood flow in microvasculature. Two transfusion therapy approaches are used: (1) simple transfusion of normal RBCs into a patient diluting the HbSS RBCs and (2) exchange transfusion which involves aphaeresis (removal) of the HbSS while transfusing normal RBCs, thus diluting HbSS containing RBCs while limiting volume fluctuation. When used chronically, exchange transfusion can prevent iron overload, stroke, and organ damage.[5,72]

Cerebral infarctions are a particular problem for patients with the SS phenotype with an average onset at 7.7 years old (most being at 5 years old) and a recurrence rate of 67%.[74] A randomized study designed by Adams *et al.* following the progression of Sickle patients undergoing transfusions aimed at keeping Hb S below 30% (with simple or exchange transfusion) showed a 93% decrease in stroke risk as compared to patients transfused only when clinically indicated.[75,76]

Due to the frequent need for transfusions, SCD patients develop antibodies against multiple antigens making compatible blood challenging to find and at times impossible to find.[71,77-82] A compiled average frequency of the most common alloantibodies in SCD patients include those against Rhesus (E, D, C) (42%), Kell (18%), Duffy (12%), and MNS (10%).[1] Vichinsky *et al.* found that out of 85 SCD children 42 were given transfusions and 10 (24%) had alloantibodies. Among the 73 adults 65 were previously transfused and 22 (34%) developed alloantibodies. The observed trend was a slight correlation between increased number of transfusions per patients and increased rates of alloimmunization. In patients with

alloantibodies, an average of 23 transfusions were given (with a range of 3 to 46 per patient); in contrast patients in whom no alloantibodies were found received on average 13 transfusions (with a range of 1 to 45). Alloantibodies found in transfused patients were against Kell (26% of transfused patients), Duffy (5.6%), and MNS (4.5%).[82] A larger study on SCD with various genotypes also found a correlation between number of transfusions and sensitization. The study included 1,205 patients less than 10 years old, 801 between the ages of 10 and 20 years old, and 925 adults over the age of 20. Adults over the age of 20 and females between 10 and 20 years old were among the most alloimmunized. This study also identified alloantibodies in 339 patients with 93 having antibodies against more than 3 different blood groups. Two patients had more than 10 different alloantibodies. Specificities for these alloantibodies also included RhD, Duffy, and MNS.[81] A possible approach to decreasing the rate of alloimmunization is through the use of ethnically matched blood due to differences in allelic frequencies of various blood groups between populations. Since the prevalence of SCD is higher in patients of African dissent, and donors are mostly Caucasian, this presents a supply problem.[83] The most common phenotype in African-Americans (found in 93%) is RhE−, RhC−, Duffy(a−), Kell−, Kidd(b−), which is found in only 7% of Caucasians. On the other hand, the phenotype RhE−, RhC+, Duffy(a+), Kell−, Kidd(b+) is found in 95% of Caucasians and in only 5% of African-Americans.[83,84] Matching blood based on ethnic background may help decrease the rate of alloimmunization in frequently transfused patients, however this would need to be done at the level of recruiting volunteer donors.

Certain **cancers** can also lead to anemia via the cancer itself or cancer treatment such as chemotherapy. Reduced RBC production can be due to decreased erythropoietin (which stimulates RBC production), decreased responsiveness of the bone marrow to erythropoietin, or stimulation of cytokines interfering with bone marrow function. Erythropoeitic therapy is offered to patients undergoing chemotherapy with Hb levels below

120g/L, which accounts for 26% of cancer patients. TchekMedyian summarized that Hb levels in 37% of oncology patients were below 120g/L at baseline and, over the course of chemotherapy treatments, an additional 41% of patients had Hb levels that low.[85]

Autoimmune hemolytic anemia (AIHA) is a unique type of anemia with the added complication of autoantibodies. AIHA is an autoimmune disease resulting in shortened native and transfused RBC lifespan via antibody binding.[1,86] Because autoantibodies are typically against common epitopes found ubiquitously on human RBCs, identification of antigen negative units for transfusion is seldom possible. Some studies have shown that autoantibodies (in addition to alloantibodies) can also arise in frequently transfused patients at a rate of 1 to 17%.[63-65]

The temperature at which these antibodies react or bind their antigen has been used as a distinguishing factor between the major types of AIHA. Warm AIHA is characteristic of IgG antibodies binding at 37^{o}C (IgA and IgM are rare). Cold AIHA autoantibodies bind antigens resulting in agglutination at 4 to 18^{o}C and are mostly of the IgM isotype. Paroxysmal cold hemoglobinuria is another AIHA subtype usually seen after a viral infection (such as Varicella) or, less commonly, after a bacterial infection. After such an infection, patients can present with hemoglobinemia, hemoglobinuria, jaundice, severe anemia and sometimes, renal failure. Occasionally, a patient will experience paroxysms of hemoglobinuria upon exposure to cold, in addition to other symptoms such as fever, back pain, leg pain, and rigors. AIHA can also be associated with a drug such as one that can bind RBC membrane (ie. Penicillin).[1,87] Generally, T cells need to give B cells the go-ahead to make antibodies, both these cells need to see the same antigen in order for that agreement to occur. This partnership is to prevent autoimmune diseases, which is more likely to occur if only one of the two cells were needed to produce an immune response. Sometimes, however, this linked recognition is deceived when two different antigens are bound together and the T cell recognizes one antigen while the B cell recognizes the other

antigen. This break in linked-recognition is thought to occur in drug induced AIHA during which the T cell identifies the bound drug as foreign and gives help to a B cell that mistakenly recognizes a self-antigen on the drug-bound RBC. In this case, the B cell goes on to produce autoantibodies.[88] Another way a drug can induce hemolysis is via the formation of immune complexes in which antibodies (usually IgM) bind multiple antigens and each antigen can bind more than one antibody. This complex can then adhere to RBCs and induce hemolysis. Methyldopa-induced AIHA has also been observed, however the mechanism of induction is unknown. A study showed dose dependent curves of methyldopa use and incidences of antibody bound RBCs in patients.[89]

Cold AIHA is usually a mild anemia and patients are advised to stay away from the cold, which can prevent exacerbation of the disease. Since human core temperature is maintained at 37^{o}C, physicians are not typically concerned about cold agglutinating antibodies. Warm AIHA however can lead to abrupt or gradual severe hemolysis. Together with hyperdynamic circulation and decrease in oxygen-carrying capacity, anemia can lead to hepatomegaly, pulmonary edema, lethargy and obtundation. Splenomegaly can also occur due to the enlargement of the white pulp (containing white blood cells). [1]

Current default therapies target macrophage dependent RBC clearance. Some therapies are aimed at controlling B cell populations. Hematocrit and Hb levels are monitored in these patients in order to avoid severe anemia. Hematocrits of less than 20% require urgent intervention. Upon such life threatening situations patients are transfused with RBCs missing the problematic antigen if the autoantibodies have been identified (in only 1-2% of cases).[1] Various Rh alleles have been found to be the culprit.[1,67,90] A summary of 8 different studies showed that out of 647 sera tested, alloantibodies were found in 209 (32%) patients with autoantibodies.[91] Patient serum is therefore tested against a panel of RBC antigens including Rhesus(D,C,E,c,e), Kell, Duffy(a,b), Kidd(a,b), and MNS(S,s). In warm AIHA, autoantibodies generally react with all RBCs tested during cross-matching making it

difficult to determine the specificity of autoantibodies.[67] Finding compatible RBC units therefore becomes particularly challenging and time consuming since the patient is already immunized against one or more sef-antigen(s).[86] As previously mentioned incompatible units may be used if the patient cardiac or cerebral functions are compromised.[1]

Risks of Transfusion therapy

In addition to potentially fatal HTRs due to incompatible transfusions, other problematic conditions can arise from either compatible or incompatible transfusions, ranging from mild to life threatening. Transfer of blood born infections is rare. Other non-infectious serious hazards (NiSHOTs) in addition to HTRs include transfusion related acute lung injury (TRALI), transfusion-associated circulatory overload (TACO), iron overload, post-transfusion purpura (PTP), and transfusion-associated graft versus host disease (TA-GvHD). Potential problems with unknown significance include transfusion-associated microchimerism (TA-MC) and transfusion related immunomodulation (TRIM).

As previously mentioned, patients needing chronic transfusions are at risk of **iron overload** (starting at 10-25g). Each RBC contains 200-500mg of iron and therefore 50-100 RBC products would be needed to reach medically harmful levels. Iron is needed for the production of sufficient RBCs due to its role in oxygen transport. Iron is also used in the electron transport chain and metalloenzyme-catalyzed reactions. Due to its Fenton chemistry reaction, iron (ferrous, Fe^{2+}) leads to the production of hydroxyl radicals (OH^-), which can attack proteins, nucleic acids, carbohydrates, and lipids.[92] Higher iron levels due to diet or excess hemolysis have been correlated with higher incidences of infections.[92-94] Ferritin (an intracellular chelator) and transferrin (an extracellular transport protein) are safeguards against free ferrous iron accumulation. During RBC turn over, macrophages clear about 20ml of RBCs and approximately 20mg of iron daily (about 1mg per hour). Each transfusion adds a bolus of RBCs that eventually is cleared by the reticuloendothelial

system (described later in this chapter), therefore increasing the levels of iron in the recipient. In animal models, iron induced pathophysiology is enhanced with the use of older RBC units because of the decreased viability observed post transfusion.[92,95,96]

Bacterial contamination occurs at a rate of about 2.6 in 100,000 RBC products transfused with a resulting reaction at rate of about 1 in 250,000. Severe reactions occur with RBCs stored for over 3-4 weeks and usually result from warm water bath contamination. Symptoms include fever (over 38.5^{o}C), chills, septic shock and can lead to death. *Yersinia enterocolitica* has been reported to be associated with RBC associated sepsis in about 60% of the cases (70% of those cases were fatal), due to its ability to proliferate at 4^{o}C. Other bacterial infections via transfusion were found to result from *Eterobacter* spp., *Campylobacter* spp., *Serratia* spp., *Pseudomonas* spp., and *Escherichia coli* contamination. In addition, *Burkholderia cepacia* and *Pseudomonas aerognosa* have been isolated from water baths. As soon as signs and symptoms of an infection are recognized, transfusion is stopped and broad-spectrum antibiotics are administered pending the identification of the organism responsible.[11]

Acute lung injury (ALI) can occur as a result of pneumonia, toxic inhalation, severe sepsis, shock, trauma, drug overdose as well as others, including **transfusion-related acute lung injury (TRALI)**. After an allogeneic transfusion, the patient may present with symptoms such as shortness of breath, non-cardiogenic pulmonary edema, fever, and hypotension. Although TRALI may be over-diagnosed due to the lack of laboratory testing, reported frequencies lie between 0.014% and 0.08% per allogeneic blood product. TRALI is most often reported when more than 60ml of plasma is present in the transfusion blood product. It is currently thought that TRALI is induced by antibodies against human leukocyte antigen (HLA) (the human major histocompatibility complex or MHC) or anti-human neutrophil antigen (HNA) present in the plasma. Once transfused, these antibodies may form leukocyte agglutinates, which pool in the lungs activating the local complement system

as well as cytokine secretion. Multiple mechanisms of TRALI appear to exist, as some reported cases resulted from transfusion of autologous units of blood, which had no detectable autoantibodies. TRALI can be fatal (5-10% of cases), although typically TRALI lasts only 48-96 hours and rarely over 7 days.[11]

Graft versus host disease (GvHD) is a consequence of white blood cells, from the transplanted tissue or transfusion, reacting against recipient cells. White blood cells causing **Transfusion associated graft versus host disease (TA-GvHD)** react mostly against HLA rich tissues like the skin, liver, and gastrointestinal tract. The reaction against the host bone marrow results in pancytopenia making this disease nearly 100% fatal. Radiation of blood products has been used as a preventative measure. Reduced TA-GvHD have been reported in the UK (in 1999) after leukoreduction was performed nationally.[11] For reasons that are unclear, TA-GvHD is much more fulminant and more often fatal than bone marrow transplant (BMT) associated GvHD. Patients with TA-GvHD are also less responsive to therapy than patients with BMT-associated GvHD.

Blood transfusions can have effects on the recipient's immune system, called **transfusion related immunomodulation (TRIM)**, which can be harmful or beneficial. Transfusion prior to transplant has been shown to increase transplanted graft survival in animals and humans,[97,98] although the opposite effect of induced rejection has also been demonstrated.[99] While members of the field continue to debate the immunomodulatory effect of transfusions, the proposed mechanism may be a simultaneous enhancement and suppression of two different branches of the immune system. Transfused RBCs may at the same time enhance B cell responses and suppress the T cells' ability to respond.[11]

As previously mentioned **Hemolytic transfusion reactions (HTRs)** are life-threatening reactions with signs and symptoms of increased red cell destruction during a transfusion. HTRs can occur immediately (acute) or over the course of a few days (delayed).[1,5] Non-antibody mediated reactions can also occur during a transfusion due to

thermal, mechanical, and osmotic stress induced on donor red cells.[5] The clinical manifestations of acute HTRs include hemoglobinurea, shock, disseminated intravascular coagulation (DIC) or bleeding, hemoglobinemia, respiratory distress, fever, renal failure, chills, abdominal/back pain, tachycardia, agitation, hypertension, and seizure.[100,101]

The frequency of HTRs (acute or immediate) has been reported by various investigators, however some believe that these reports do not represent the totality of HTR occurrences. An early study by Kilduffe and DeBakey in 1942 with 43,284 transfusions yielded 80 hemolytic reactions (0.18%) and 32 deaths (0.07%).[5]

In more recent years, reports have been published on blood transfusion related deaths. From 1964 through 1973, the Mayo Clinic reported 23 cases of delayed HTRs and 24 cases of acute HTRs.[102] In 1975, it became mandatory to report any blood transfusion related death to the Bureau of Biologics (BOB) of the Food and Drug Administration (FDA) within 7 days of the event;[100,103] Two articles interpreted and summarized reported transfusion related deaths between 1976 and 1978. One report concluded that 44 out of 64 deaths were due to acute HTRs.[100] The other study deduced from the reported 69 deaths that only 22 could be ascribed to acute HTRs.[103] Both groups agree that 2 delayed HTRs were reported within this period of time and that erroneous transfusions were most often due to blood given to the wrong patient.[100,103] Reported symptoms of acute HTRs most frequently included fever, respiratory distress, hemoglobinuria, and hypotension or shock.[100] Based on the number of transfusions in 1978 (9.4 million units of blood), the number of transfusions in the 3 years studied was estimated to be around 27 million units with a total of about 64 transfusion-related fatalities, a rate of about 1 per 422,000 units.[100] It was estimated that 9 deaths would have occurred regardless of transfusion.[103]

Sazama later published a more extended summary of all reported transfusion related deaths between the years of 1976 and 1985. Out of the 355 reported deaths, 158 (37%) were due to acute HTRs (by far the most common) and 26 were due to delayed HTRs

(7.3%). Delayed HTRs occurred between 6 to 16 days and were reported to be predominantly against RhD (34%), Kidd (30%), Duffy (14%), Kell (13%), and MNS (4%) blood group systems.[104-106] The most common signs reported included (in order of decreasing frequency) hemoglobinurea, disseminated intravascular coagulation, hemolysis, renal failure, and hypotension. A positive DAT was mentioned in 20 reports.[104]

More recently, in 1992, Linden *et al.* reported a total of 104 significant errors (an incidence of 1/19,000) in the state of New York from 285 regulated facilities between January 1, 1990 and October 31, 1991. About half (52%) were due to ABO incompatibility (an incidence of 1/33,000), 34% occurred with ABO compatible RBCs (potentially against other blood groups) and 4 cases were due to Rh incompatibility (an incidence of 1/52,000).[107] Linden *et al*. later published a more extensive study on erroneous administration of RBCs from 1990 to 1999. The frequency of ABO-incompatible RBC transfusion was 1/38,000, of ABO-compatible RBCs was 1/41,000, and of total fatal cases was about 1 per 1.8 million (5 deaths, or 4% of reported acute HTR cases). It was again concluded that the majority of errrors was due to misidentification of the patient.[108]

The FDA published a report in 2010 on transfusion related deaths from the fiscal year of 2005 to 2010. Of the total reported fatalities (307), 24% was due to HTRs resulting from ABO incompatibility (9%) and non-ABO incompatibility (15%) with 6% due to anti-Duffy antibodies. A decrease in reported HTRs was observed in 2001.[109]

Hemolytic Disease of the Fetus and Newborn (HDFN)

Another life-threatening reaction induced by antibody binding of RBCs is HDFN. It is most commonly observed with the Rh antigen if the preventative treatment with Rhogam (anti-human Rh antibodies) is not used. It is unclear why Rhogam is so effective in preventing Rh alloimmunization via pregnancy. Philip Levine in the 1930s[5] observed post transfusion hemolysis during an O blood transfusion to an O patient and concluded that

ABO typing might not be sufficient for compatible transfusions. Levine later noticed agglutination of the O-type donor blood when added *in vitro* to the O-type recipient serum. This phenomenon was due to the Rh blood group, which was so named by Landsteiner and Alex Weiner who both performed experiments with serum from animals immunized with Rhesus blood. They noticed that this serum caused agglutination in 85% of humans tested, who became known as Rh-positive. The Rh blood group has a multitude of Rh variants (or Rh antigens), one of which is called the D antigen. RhD is considered the most immunogenic of the Rh blood group antigens[1] and induces the second most clinically significant antibodies behind ABO, causing both HTRs and HDFNs.[11] Presence or absence of RhD is referred to as Rh-positive (or just "+") or Rh-negative (or just "–"), respectively, along side of the individual's ABO blood group letter designation.[1] So far, 45 antigens on two 12-pass transmembrane proteins (RhD and RhCE) have been noted. Most of the Rh antigens elicit an IgG response posing a particular problem during pregnancy (some will also induce IgM, and rarely IgA).[3]

Due to their clinical significance, anti-D antibodies are particularly problematic for Rh-negative mothers pregnant with an Rh-positive child. The fetus can develop a positive DAT from the mother's circulating anti-D antibodies by the eighth week of pregnancy. As a result, the fetus can suffer severe anemia and death *in utero*.[5] Transferring antibodies from mother to child *in utero* is only possible through an active process via a neonatal Fc receptor (FcRn) found on the placenta. FcRn selectively binds IgG for cross-placental transport maintaining high levels of IgG in neonatal plasma throughout intrauterine life.[88] Anti-D alloantibodies can become detectable during the mother's first pregnancy or after giving birth to their first-born.[5,110] A study by Hartmann and Brendemoen included serological tests of 75,000 pregnant women in addition to blood typing tests of fathers. They found that 63 women were immunized via transfusion, 23 were immunized as a result of a 1st pregnancy and 434 were immunized as result of multiple pregnancies.[111] Treatments with anti-RhD antibodies have

been, and are still, used during pregnancy to prevent immunization of the mother against the Rh D+ baby.[112]

Age-dependent RBC clearance mechanisms

RBC lifespan is about 115-120 days in humans and 50-60 days in mice. Changes on the RBC surface are understood to be a potential sudden trigger for removal/death. RBC age and death is thought to be due to alterations associated with decreased metabolic activity, changes in cell shape, membrane modifications, oxidative injury, microvesiculation and exposure of removal markers.[113] Older RBCs have been shown to have more surface bound antibodies[114] and are preferentially phagocytosed[115] as compared to a younger RBC population.

RBCs have no nucleus and lack mitochondria; thus, once an RBC leaves the bone marrow no new protein synthesis occurs; therefore, an RBC has only a finite amount of proteins which can degrade or become modified overtime. This degradation exposes neoantigens, which can be recognized by circulating antibodies. Phosphorylation, oxidation and aggregation of proteins have been implicated in regulating RBC homeostasis and lifespan.[113] Protein 4.1 and Band 3 clustering have been considered aging determinants of RBCs. A strongly held model of self-reactive antibodies labeling degraded RBC surface proteins is thought to be another RBC aging process, although there remains some controversy. Degraded and/or clustered Band 3 protein products are potential targets for such antibodies, targeting the 'aged' RBC to the reticuloendothelial system for removal.[113,116] Band 3 degradation has also been observed with stored RBC units. The increased number of new epitopes associated with RBC age was correlated with *in vivo* autoantibody recognition.[117]

Oxidative stress also causes damage to the RBC expediting its death. Vitamin E seems to protect RBCs from oxidative damage. RBCs from vitamin E deficient animals have

been shown to correlate with premature aging of the RBC, based on susceptibility to phagocytosis, IgG binding and anion transport ability. Increased Band 3 break down products were also observed in RBCs from these animals. The authors suggested that the increased number of oxidative damaged RBCs (with no vitamin E present) are more susceptible to clearance and mimic older RBC populations.[118]

Older RBCs were also shown to be denser than younger RBCs. Vesicles containing Hb have also been isolated from the blood and is thought to be an age-dependent process leading to smaller RBCs. This process diminishes the RBC stock of Hb and, with a disproportionate loss of membrane, the RBC becomes denser and more rigid. Its decreased deformability is also believed to be a sign of RBC maturation.[113,119,120] The splenic architecture is such that RBCs must squeeze through tight spaces in order to return into the circulation. If RBCs are unable to do this, they stay sequestered by the spleen in a macrophage rich nitch. The number of dense RBCs has been shown to be exacerbated in asplenic individuals.[121] RBC removal via the reticuloendothelial system is discussed in more detail in the next section.

Membrane alterations leading to phosphotidylserine (PS) exposure on the outermembrane rather than intracellularly has also been considered a mechanism of RBC aging. Cells with high PS levels on the surface can be recognized by phagocytic cells and are typically rapidly removed from the circulation.[88,122,123] Willekens *et al.* also showed that RBC Hb vesicles can be taken up by liver phagocytes (Kupffer cells) in a PS and scavenger receptor ligand dependent manner.[124] Another group further demonstrated that macrophages have receptors (stabilin1 and 2) that mediate clearance of beads labeled with PS.[123] Many of these progressive changes have been observed in stored RBCs.[125]

Eryptosis is also a mechanism of RBC clearance and may be distinct from gradual progressive RBC aging. It bears many similarities to apoptosis, however RBCs do not have a nucleus. The process can be initiated by hyperosmotic shock and Cl^- removal. Once

eryptosis is triggered prostoglandin E_2 (PGE_2) is released, activating nonselective cation channels, which in turn increases intracellular calcium (Ca^{2+}). This sudden Ca^{2+} influx triggers PS exposure on the outer membrane.[126]

Antibody-dependent RBC clearance mechanisms

As previously mentioned, preemptive RBC death can occur during incompatible transfusion, autoimmune disease, and HDFN. The main RBC clearance mechanisms mediated by antibodies are through the complement cascade (intravascular hemolysis) and Fc receptors (extravascular hemolysis).[5,127]

The **complement system** is a series of proteolytic events leading up to the formation of a membrane attack complex (MAC), compromising the target's membrane and osmotic homeostasis. Complement can be triggered by one of 3 ways called the classical pathway (via antibody binding), the lectin pathway (via lectin binding), and the alternative pathway (via spontaneous activation of complement protein C3). The classical pathway can be activated by IgM and IgG3, to a lesser extent IgG1, and to an even lesser extent IgG2.[88] Binding of antibodies to certain blood groups have been known to activate complement, including anti-Kidd (50-70% of the time), anti-A or anti-B (usually), anti-Kell (sometimes), and anti-Duffy (sometimes). Rhesus, however has not been shown to activate complement[127] but rather was shown to be involved in antibody mediated phagocytosis (discussed later in this section).[128]

The classical pathway starts with the activation of C1 by antibody(s) (IgM or 2 IgGs). Once enzymatically active, C1 cleaves C4 and C2. Lectins do not involve the C1 subunits and start the cascade at C4 instead. Next, cleaved C4 and C2 subunits combine to cleave C3. At this point all pathways follow the same cascade, since the alternative pathway is spontaneously activated via C3 degradation. Additional proteolytic proteins are involved in amplifying numbers of available C3 convertases. Cleaved C3 forms a C5 convertase

complex, cleaving C5 into C5b, which later forms MAC with C6, C7, C8, and C9. Once formed, the MAC complex inserts itself into the membrane of the target cell. The newly formed pore disrupts cellular osmotic homeostasis, resulting in cellular death, which can occur intravascularly.[88,127] C5 can also be directly activated by thrombin.[129] In patients with paroxysmal nocturnal hemoglobinuria (PNH), treatment with anti-C5 antibodies abrogated intravascular hemolysis and showed increased numbers of C3-coated RBCs with evidence of continued RBC clearance.[130] Animals deficient for C4 have shown near normal survival of transfused incompatible RBCs as compared to control animals, in which incompatible RBCs cleared.[131,132]

In addition to their ability to directly lyse cells intravascularly, complement proteins can also bind complement receptors (CR). When bound, complement receptors can trigger a variety of actions including phagocytosis, erythrocyte transport of immune complexes, and antibody production. Activated C3 and further degraded products can bind most of these receptors. During the complement cascade, activated C3 exposes its highly reactive thioester bond, which can attach the RBC surface and the RBC bound antibody. Once bound, C3b can either form a C5 convertase (leading to the formation of a MAC complex) or further degrade to iC3b and C3dg. Each of these C3 variants can bind a different complement receptor present on various white blood cells.[133] The rate of degradation can vary according to where C3b is bound and antibody presence. Degradation rates of C3 bound to the Ig heavy chain seems to be slower than of free C3.[134]

C3b has the highest affinity for CR1 and CRIg. iC3b can bind CR2, CR3, CR4 and CRIg. Finally, C3dg associates mostly with CR2.[135] CR1 (CD 35; Knops blood group when found on RBCs) has a high affinity for both C3b and C4b with lower affinities for iC3b and C3dg.[136] CR1 often has an important role in mediating phagocytosis and capturing immune complexes on erythrocytes. The immune complex is later removed in the liver via CR1 without destroying the RBC that carried it there.[87,127,135] CR1 also has a negative feedback

role of inhibiting the complement cascade through enhancing proteolytic cleavage of C3b[137,138] and inhibiting C3 convertase.[139] CR2 (CD 21) is present on B cells which can uptake the RBC coated with C3 degraded products leading to differentiation and enhancement of B cell mediated immunity.[127,135] CR3 and CR4 (CD 11b and CD 11c, respectively) are involved in cell trafficking, costimulation and synapse formation, in addition to phagocytosis. CR3 is found on epithelial cells, neutrophils, monocytes, natural killer cells, and cells that mediate antibody-dependent cell cytotoxicity (ADCC).[127] CRIg (aka. Z39Ig and VSIG4) is the most recently discovered complement receptor and regulates T cell proliferation. CRIg has been found on resident macrophages, such as Kupffer cells, interstitial macrophages in the heart, and synovial lining macrophages, however not on circulating cells.[135]

Various non-complement proteins can regulate the function of complement. Decay-accelerating factor (DAF; aka. CD55) prevents accelerated decay of C3 and C5 convertases. MAC-inhibitory protein (MIP; aka. C8 binding protein and homologous restriction factor) inhibits MAC formation. Protectin (aka. HRF20, P18, and MAC-inhibitory factor) also inhibits MAC on homologous cells. The function of these proteins was revealed in paroxysmal nocturnal hemoglobinuria (PNH), during which RBCs are deficient in these complement regulators and intravascular hemolysis ensues.[127]

Complement can be depleted from experimental animals in various ways. Older methods utilize heat aggregated human immunoglobulin (complement consumption assays)[140,141] or cobra venom factor.[142] Cobra venom factor was found to be efficient in depleting the host of C3 and C5 (nearly 100%) as well as diminishing levels of C6, C7, C8 and C9 (60-70%) in the host.[142] Other means of complement removal are available today such as the use of antibodies against complement components (as previously mentioned) and animals knocked out for a specific complement component.

The second major mechanism understood to be responsible for incompatible RBC clearance is mediated by **Fc receptors**, leading to phagocytosis, which is typically but not always extravascular. Differences between humans and the murine model organism do exist however. The IgG subtypes IgG1, 2, 3, and 4 are found in humans, whereas IgG 1, 2a, 2b, 2c, and 3 are found in mice. Three types of Fcγ receptors exist in both species. Both human and mouse FcγRI (CD 64) receptors are found on macrophages. The mouse FcγRI binds most tightly to IgG2a whereas the human form favors IgG1 and 3. FcγRII (CD 32) is an inhibitory receptor in mice with the highest affinity for IgG1, IgG2a, and IgG2b. In humans, there are 2 types of FcγRII receptors, an activating (RIIa) and an inhibitory (RIIb) receptor with binding affinities to IgG1 and IgG3. FcγRIII (CD 16) receptors in both species are found on phagocytes. IgG1 and 3 bind with equally high affinities to FcγRIII in humans, however in mice IgG1, IgG2a, and IgG2b bind most tightly to FcγRIII. Two forms of FcγRIII are found in humans. FcγRIIIa is transmembrane and resembles the mouse FcγRIII, however the human FcγRIIIb is GPI-linked and is not found in murine animals.[143] The common γ chain is a signaling molecule associated with FcγRI and FcγRIII (in both species) and also with FcγRIV, which was found in mice but not in humans.[88,144]

Macrophages known to be involved in RBC clearance are part of the **reticuloendothelial system (RES).** The RES is a mononuclear system mostly located in the reticular connective tissue of the spleen, liver, lungs, bone marrow and, to a lesser extent, lymphoid tissues. A more accurate nomenclature was later developed: mononuclear phagocytic system (MPS). The name RES was based on the inaccurate understanding that macrophages derive from endothelial cells (macrophages are mesenchymal) and that these cells secret collagen-like substances, which is not the case. Despite the misnomer, RES is still widely used today.[127]

The liver and the spleen were shown to be responsible for the majority of the clearance of transfused incompatible RBCs.[145] Moreover, a human study showed that transfused incompatible Cr^{51}-labeled RBCs accumulated in the spleen as they were gradually eliminated from circulation.[146] In addition to ridding the blood stream of microorganisms, particles and debris, the spleen also removes aged, abnormal, antibody-bound, and complement sensitized RBCs from the circulation.

The spleen also houses white blood cells (largely found in the white pulp) to fight off blood born pathogens and filters RBCs in the red pulp, which has an exceptionally high concentration of RBCs. Its architecture facilitates extended RBC contact with resident macrophages. As part of their circulation, RBCs flow through the intricate splenic vasculature. Once the terminal arteries dump RBCs in the red pulp, these cells percolate through macrophage-rich filter beds. In order to reenter the circulation, RBCs must be capable of squeezing through the sinusoidal space. If deformed or too rigid, RBCs stay in the filter beds, mentioned earlier as a mechanism of aged RBC removal.[127]

The liver is another organ that is responsible blood filtration of pathogens and RBC anomalies. It contains the largest group of fixed macrophages in the body, called Kupffer cells. These cells are the first to encounter pathogens that would have made their entrance through the gut. Kupffer cells have been found to be very important in RBC clearance as well.[147] However, unlike the spleen, the liver does not have a paranchymal filtration structure.

For patients requiring transfusions exceeding 200ml packed RBCs per kg body weight, physicians suggest splenectomies to help decrease transfusion load.[57] It has also been used to increase RBC survival levels in patients with AIHA.[1,86] Splenectomies were shown to increase survival in sensitized RBCs in guinea pigs to near normal levels.[145] This surgery however has not always yielded increased RBC survival. It had little effect on RBC survival in patients treated for cryptogenic splenomegaly (with and without liver cirrhosis).

RBCs of splenectomized patients were found to be more sensitive to osmotic changes. In addition, patients with liver cirrhosis had higher transfused RBC survival than patients with no liver cirrhosis, prior to splenectomy.[148]

Both the liver and the spleen have been shown to be involved in RBC clearance. A study showed that prior to splenectomy, the majority of incompatible RBCs accumulated in the spleen (with little seen in the liver), however after splenectomy, the liver amassed nearly the same level of incompatible RBCs as the spleen in unsplenectomized hosts.[149] A study by Mollison and Hughes-Jones tracked the area of transfused incompatible Cr^{51}-RBCs accumulation as a function of clearance kinetics, using antibodies to various antigens. In eight cases, during which clearance occurred rapidly (half time of 2-6 min), most of the radioactivity settled in the liver (74%) versus the spleen (20%). Whereas cases in which it took more than 15 minutes (some cases were 56-75 minutes) to clear half of the transfused RBCs, the spleen accumulated most of the radioactivity (some 60-102%) and the liver only had 7-26% of the recovered radioactivity.[150]

In some instances, RBCs return to circulation after a **temporary sequestration**. This is thought to occur as a result of complement deposition and attachment to macrophages via C3b and iC3b. Once iC3b is cleaved to C3dg, the RBC is released back in the circulatory system.[5] A study showed an antibody dose-dependent temporary sequestration of RBCs. A high enough antibody dose was needed for the permanent removal of RBCs, otherwise the RBCs would return into circulation.[127] A study by Jandl *et al.* showed almost immediate clearance of sensitized radiolabeled RBCs, which gradually returned to circulation over the course of days (up to 90% were recovered).[149] Other studies have also shown rapid initial clearance with subsequent reappearance of labeled incompatible RBCs after 3-4 hours[131,151] with one study only showing minor recovery after 24 hours.[132]

Many studies on **RBC clearance kinetics** were done between the 1950's and 1980's. Cutbush and Mollison performed a series of incompatible transfusions in humans

with RBCs expressing a variety of blood group antigens (Kell, Rhesus, Kidd, Duffy, and more). Transfusions were performed with presensitized RBCs or with untreated RBCs introduced into incompatible hosts. In both types of transfusions, an initial rapid clearance was observed within minutes of injection followed by a slower clearing kinetic (in certain cases, no additional clearance was observed).[152] Rapid initial clearance was observed with transfusions of RBCs into incompatible hosts with anti-Kell, anti-Duffy(a) (also with pre-sensitized RBCs), and anti-M of MNS.[149] RBC clearance in the presence of anti-RhD was found to occur in a matter of minutes with a steady decline to less than 5% of recovered Cr^{51} 1 hour post transfusion in healthy individuals. After two months of treatment with cortisone (a cortico-steroid hormone), one patient had higher Rh-sensitized RBC survival 40 minutes post incompatible transfusion (about 30% continued to circulate while none were detectable in untreated individuals). Very little to no difference in survival of incompatible Rh-RBCs was observed, however, in four patients treated with corticosteroids.[150,153]

Incompatible RBC rate of clearance was found to be relative to the dose of antibody used. Individuals passively immunized with increasing amounts of anti-RhD antibodies induced increasing levels of RhD+ RBC clearance.[154] The same post transfusion survival trend was observed when pre-incubating RBCs with increasing levels of anti-RhD antibodies.[155] Other antigens have also shown dose-dependent clearance, such as Duffy(a) and A_1 of ABO.[155,156] Increased clearance was observed in individuals passively immunized with the same dose of anti-A_1 antibodies and transfused with decreasing amounts of A_1 RBCs.[156]

As previously discussed antibodies can mediate clearance via Fc receptors or via the complement cascade. Antibodies are not known to be directly damaging to the bound RBC.[5] However, Brain *et al*. suggested that antibodies bound to the surface of incompatible RBCs generated mechanical stress, which opened Ca^{2+} channels leading to Ca^{2+} influx and RBC death.[157-159] Other animal studies have suggested Fc receptor and complement independent

mechanisms of clearance. Anti-A IgG antibodies were shown to directly trigger PS exposure *in vitro*.[160] Moreover, RBC agglutination was observed as a mechanism coinciding with clearance in a murine model of AIHA (NZB mice).[161]

Research on RBC incompatibility has mostly yielded RBC survival data, with little information on antibody isotype. IgG1 was found to be a potent inducer of hemolysis,[162] however, this was contradicted by a later study, which found no such effect.[163] Starting around the 1950s, research on incompatible transfusions was first performed on mice and humans. Until the 1980s, human subjects were given incompatible transfusions in order to study RBC survival and clearance, although recipients were given polyclonal anti-serum. Since around the 1980s, data on human incompatible transfusions have been retrospective incompatible transfusions (with very little from clinical trials). Studies on RBC incompatibility generally do not take into account the antibody isotype involved in RBC removal. Recently, some studies have utilized monoclonal antibodies and the mouse model to understand the affect of antibody isotype on incompatible RBCs and the host.[164-166] Little is understood about RBC clearance and the isotype involved.

RBC survival in the presence of antibody

Despite circulating in a host with antibodies present, RBCs do not always clear. It has been observed that when A and B (of the ABO blood group) incompatible RBC clearance is rapid, the majority are eliminated (99.9%), but if clearance is slower some RBCs survive long term and presumably have acquired resistance to the transfusion reaction.[5] Close to 20% of transfused O and A_2 RBCs in an A_1 recipient containing anti-O antibodies have been observed to continue circulating after 2-5 hours.[155] Similar trends were observed with other blood groups. Rapid clearance (within 15 minutes) was initially observed with Lewis(b) incompatible RBCs, however 50% did not clear, when survival was followed up to 6 hours.[167] A similar observation was made with P+ RBCs (of the Globoside

blood group) in patients with anti-P serum. Some patients cleared as much as 50% of the incompatible blood in 20 minutes and the rest survived up to an hour with no further clearance.[152] Pre-incubating Duffy(a) RBCs with anti-Duffy(a) antibodies also lead to long term survival of a portion of the RBC population but this was no longer observed if the concentration of the anti-Duffy(a) antibody was doubled.[155] It has also been shown that IVIG injections increase the survival of IgM sensitized RBCs in guinea pigs.[134] CD 47, a Rhesus associated antigen, was shown to prevent erythrophagocytosis in the spleen.[113,119,168]

The antibody classes in these studies have not been determined, which can play a role in limiting clearance. Some antibodies that are not expected to be problematic during an incompatible transfusion are (1) active only at temperatures below 37^{o}C, (2) are active at that temperature but are IgMs that do not bind complement or (3) are of the IgG1 (not always), IgG2, or IgG4 subclass. Even though observations have lead to these conclusions, it is advised to continue cross-matching donor blood and recipient serum for clinically significant blood groups.[5]

In certain cases of HDFN, the fetus of an RhD negative mother continues to develop and the infant is born alive with a positive DAT. Hemolysis still occurs and is maximal at the time of birth, however declines overtime. Many infants with positive DATs have no increase in RBC destruction.[5] A lack of HDFN reaction was also observed with antibodies to various other blood group antigens such as Scianna (Sc1), Dombrock (Do^{a}, Gy^{a}, Hy), Gerbich (Ge2, Ge3), Er (Er^{a}), Vel, At^{a} and jr^{a}.[3] Due to the lack of reaction elicited, these antibodies are considered to be clinically insignificant in the context of HDFN.

Complement components and antibodies have also been found on RBCs of normal individuals with no observable ailments.[127] Chaplin *et al.* concluded that 50 to 160 C3d molecules were bound to each RBC in normal individuals.[169] Another report by Merry *et al.* found about 420±140 C3 molecules per RBC.[170] C4 molecules were also observed on normal RBCs, in addition to C3 and C3d.[171] Moreover, about 39±20 IgG molecules per RBC

(washed) have also been revealed.[172] When comparing the level of bound IgGs on the surface of washed RBCs from healthy individuals to washed RBCs from AIHA patients, more IgGs were found on AIHA RBCs however RBCs from healthy individuals also had bound antibodies.[173]

Work has been done to understand these anomalies. In a mouse model, Göran Möller transfused RBCs incompatible to the subsequent injections of H-2 (mouse Major Histocompatibility Complex (MHC)) antibodies. In this case some clearance was observer after the first anti-H2 injection, however no more clearance occurred after additional injections of the anti-H2.[174] In a following manuscript, Möller determined that the continued survival ("resistance") of incompatible RBCs was due to complement, by treating mice with heat-aggregated Igs (used to deplete host of complement).[175]

Another process in which incompatible RBCs can avoid their fate of hemolysis is via **antigen loss** (also named weakened antiginicity). This event has most often been observed in AIHA patients with an initial identification of an antibody against a blood group antigen and an RBC null phenotype with regard to that antigen, as well as an initial negative DAT. These observations were then followed by a reappearance of the antigen in question on the patient's RBCs, therefore the antibody detected was an autoantibody. Antigen loss is most frequently observed with the Kell blood group however it has also been observed with other blood group systems of various membrane biochemistries. Antigen loss has been observed in single-pass transmembrane proteins (Kell, Kidd, LW, Gerbich, AnWj), multipass transmembrane proteins (Rh), and GPI-linked proteins (Cromer).[176] Antigen loss was first demonstrated in a murine model[177] and was found to require at least 2 different monoclonal antibodies, which simultaneously bind 2 different epitopes on the same RBC protein.[178] RBCs do not have a nucleus and therefore do not produce any additional surface antigens, making the study of antigen loss possible with the use of these cells.

Models for transfusion research

Transfusion research has been performed in a variety of model organisms and in humans. In the past, RBCs were labeled prior to transfusion using the ^{51}Cr radioactive isotope.[179] In order to determine the survival of the transfused RBCs, samples were collected and RBCs were lysed in order to quantify the level of radioactivity in the sample. Some human studies by Mollison were done using an additional radioisotope (^{32}P) to label a control RBC population.[150,180] Human studies of incompatible transfusions are not as easily approved these days, due to the ethical repercussions. Therefore, human contemporary publications are typically solely observational, or are part of a clinical trial. A few mouse models (to date) have been used to study hemolytic transfusion reactions, two of which are used in this dissertation.[164,165] As shown by this review of the literature, most studies on incompatible transfusion, human work and animal work alike, have been done in the last century. Despite the endless testing done by the blood banks, incompatible transfusions still occur (as I have discussed earlier) and our understanding of the field is still very limited.

Contemporary tools, such as flow cytometry, have helped us get a closer look at RBCs. With the use of two blood group antigens and three antibodies, I attempt to shed some light on the biology of incompatible transfusions.

Hypothesis 1: *The clearance mechanisms used by anti-glycophorin A and anti-Duffy antibodies are via complement or Fcγ receptors.*

Hypothesis 2: *Post incompatible transfusion survival of glycophorin A RBCs requires complement.*

Hypothesis 3: *Post incompatible transfusion survival of Duffy RBCs requires complement.*

References

1. *Hillyer CD SL, Ness PM, Anderson KC, Roback JD, editor Blood Banking and Transfusion Medicine. Second ed. Philadelphia: Churchill Livingstone, 2007.*
2. *Ottenberg R, Kaliski DJ. Accidents in Transfusion. JAMA 1913;**61**: 3.*
3. *Reid ME, Lomas-Francis C. The Blood Group Antigen FactsBook. London: Academic Press, 1997.*
4. *Reid ME, Mohandas N. Red blood cell blood group antigens: structure and function. Semin Hematol 2004;**41**: 93-117.*
5. *Klein H G ADJ, editor Mollison's Blood Transfusion in Clinical Medicine Victoria, Australia: Blackwell Publishing, 2005.*
6. *Daniels GL, Moulds JJ, Anstee DJ, Bird GWG, Brodheim E, Cartron JP, Dahr W, Engelfriet CP, Issitt PD, Jørgensen J, Kornstad L, Lewis M, Levene C, Lubenko A, Mallory D, Morel P, Nordhagen R, Okubo Y, Reid M, Rouger P, Salmon C, Seidl S, Sistonen P, Wendel S, Woodfield G, Zelinski T. ISBT Working Party on Terminology for Red Cell Surface Antigens: São Paulo Report. Vox Sanguinis 1993;**65**: 77-80.*
7. *Reid M, Yahalom V. Blood Groups and Their Function. Baillière's best practice & research. Clinical haematology 2000;**13**: 485-509.*
8. *Janatpour KA, Kalmin ND, Jensen HM, Holland PV. Clinical outcomes of ABO-incompatible RBC transfusions. Am J Clin Pathol 2008;**129**: 276-81.*
9. *Coombs RR, Mourant AE, Race RR. A new test for the detection of weak and incomplete Rh agglutinins. Br J Exp Pathol 1945;**26**: 255-66.*
10. *Yamamoto F. Molecular genetics of the ABO histo-blood group system. Vox Sang 1995;**69**: 1-7.*

11. *Hillyer CD SB, Zimring JC, Abshire TC, editor Transfusion Medicine and Hemostasis. First ed. Burlington: Elsevier, 2009.*

12. *Greenwell P. Blood group antigens: molecules seeking a function? Glycoconj J 1997;**14**: 159-73.*

13. *Springer GF, Horton RE. Blood group isoantibody stimulation in man by feeding blood group-active bacteria. J Clin Invest 1969;**48**: 1280-91.*

14. *Andersson M, Carlin N, Leontein K, Lindquist U, Slettengren K. Structural studies of the O-antigenic polysaccharide of Escherichia coli O86, which possesses blood-group B activity. Carbohydr Res 1989;**185**: 211-23.*

15. *Miller LH. Impact of malaria on genetic polymorphism and genetic diseases in Africans and African Americans. Proc Natl Acad Sci U S A 1994;**91**: 2415-9.*

16. *Tomita A, Radike EL, Parker CJ. Isolation of erythrocyte membrane inhibitor of reactive lysis type II. Identification as glycophorin A. J Immunol 1993;**151**: 3308-23.*

17. *Anderson RA, Marchesi VT. Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide. Nature 1985;**318**: 295-8.*

18. *Auffray I, Marfatia S, de Jong K, Lee G, Huang CH, Paszty C, Tanner MJ, Mohandas N, Chasis JA. Glycophorin A dimerization and band 3 interaction during erythroid membrane biogenesis: in vivo studies in human glycophorin A transgenic mice. Blood 2001;**97**: 2872-8.*

19. *Anderson RA, editor Red Blood Cell Membranes: Dekker, 1989.*

20. *Luna EJ, Hitt AL. Cytoskeleton--plasma membrane interactions. Science 1992;**258**: 955-64.*

21. *Bloch RJ, Pumplin DW. A model of spectrin as a concertina in the erythrocyte membrane skeleton. Trends Cell Biol 1992;**2**: 186-9.*

22. *Chasis JA, Mohandas N. Red blood cell glycophorins. Blood 1992;**80**: 1869-79.*

23. *Tanner MJ. The major integral proteins of the human red cell. Baillieres Clin Haematol 1993;**6**: 333-56.*

24. *Chasis JA, Mohandas N, Shohet SB. Erythrocyte membrane rigidity induced by glycophorin A-ligand interaction. Evidence for a ligand-induced association between glycophorin A and skeletal proteins. J Clin Invest 1985;**75**: 1919-26.*

25. *Chasis JA, Mohandas N. Erythrocyte membrane deformability and stability: two distinct membrane properties that are independently regulated by skeletal protein associations. J Cell Biol 1986;**103**: 343-50.*

26. *Chasis JA, Reid ME, Jensen RH, Mohandas N. Signal transduction by glycophorin A: role of extracellular and cytoplasmic domains in a modulatable process. J Cell Biol 1988;**107**: 1351-7.*

27. *Chasis JA, Schrier SL. Membrane deformability and the capacity for shape change in the erythrocyte. Blood 1989;**74**: 2562-8.*

28. *Andersson LC, Gahmberg CG, Teerenhovi L, Vuopio P. Glycophorin A as a cell surface marker of early erythroid differentiation in acute leukemia. Int J Cancer 1979;**24**: 717-20.*

29. *Kina T, Ikuta K, Takayama E, Wada K, Majumdar AS, Weissman IL, Katsura Y. The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. Br J Haematol 2000;**109**: 280-7.*

30. *Cutbush M, Mollison PL, Parkin DM. A New Human Group. Nature 1950;**165**: 2.*

31. *Goodrick MJ, Hadley AG, Poole G. Haemolytic disease of the fetus and newborn due to anti-Fy(a) and the potential clinical value of Duffy genotyping in pregnancies at risk. Transfus Med 1997;**7**: 301-4.*

32. *Miller LH, Mason SJ, Dvorak JA, McGinniss MH, Rothman IK. Erythrocyte receptors for (Plasmodium knowlesi) malaria: Duffy blood group determinants. Science 1975;**189**: 561-3.*

33. *Hadley TJ, Peiper SC. From malaria to chemokine receptor: the emerging physiologic role of the Duffy blood group antigen. Blood 1997;**89**: 3077-91.*

34. *Neote K, Mak JY, Kolakowski LF, Jr., Schall TJ. Functional and biochemical analysis of the cloned Duffy antigen: identity with the red blood cell chemokine receptor. Blood 1994;**84**: 44-52.*

35. *Lefrere JJ. Transfusion medicine history illustrated. Arming for war: the donor's arm. Transfusion 2011;**51**: 2064-5.*

36. *Beers MH, Berkow R. The Merck Manual of Diagnosis and Therapy: Merck Research Laboratories 1999.*

37. *Dahlke MB. Red blood cell transfusion therapy. Med Clin North Am 1984;**68**: 639-56.*

38. *Rasanen J. Supply-dependent oxygen consumption and mixed venous oxyhemoglobin saturation during isovolemic hemodilution in pigs. Chest 1992;**101**: 1121-4.*

39. *Weiskopf RB, Viele MK, Feiner J, Kelley S, Lieberman J, Noorani M, Leung JM, Fisher DM, Murray WR, Toy P, Moore MA. Human cardiovascular and metabolic response to acute, severe isovolemic anemia. JAMA 1998;**279**: 217-21.*

40. *Wilkerson DK, Rosen AL, Sehgal LR, Gould SA, Sehgal HL, Moss GS. Limits of cardiac compensation in anemic baboons. Surgery 1988;**103**: 665-70.*

41. *Fullerton WT, Turner AG. Exchange transfusion in treatment of servere anaemia in pregnancy. Lancet 1962;**1**: 75-8.*

42. *Carson JL, Poses RM, Spence RK, Bonavita G. Severity of anaemia and operative mortality and morbidity. Lancet 1988;**1**: 727-9.*

43. *Spence RK, Carson JA, Poses R, McCoy S, Pello M, Alexander J, Popovich J, Norcross E, Camishion RC. Elective surgery without transfusion: influence of preoperative hemoglobin level and blood loss on mortality. Am J Surg 1990;**159**: 320-4.*

44. *Nelson AH, Fleisher LA, Rosenbaum SH. Relationship between postoperative anemia and cardiac morbidity in high-risk vascular patients in the intensive care unit. Crit Care Med 1993;**21**: 860-6.*

45. *Carson JL, Noveck H, Berlin JA, Gould SA. Mortality and morbidity in patients with very low postoperative Hb levels who decline blood transfusion. Transfusion 2002;**42**: 812-8.*

46. *Ott DA, Cooley DA. Cardiovascular surgery in Jehovah's Witnesses. Report of 542 operations without blood transfusion. JAMA 1977;**238**: 1256-8.*

47. *Hillman RS, Finch CA. Red Cell Manual. 7 ed. Philadelphia: F. A. Davis Company, 1996.*

48. *Kaferle J, Strzoda CE. Evaluation of macrocytosis. Am Fam Physician 2009;**79**: 203-8.*

49. *Paulson RF, Shi L, Wu DC. Stress erythropoiesis: new signals and new stress progenitor cells. Curr Opin Hematol 2011;**18**: 139-45.*

50. *Drews RE. Critical issues in hematology: anemia, thrombocytopenia, coagulopathy, and blood product transfusions in critically ill patients. Clin Chest Med 2003;**24**: 607-22.*

51. *Galanello R, Origa R. Beta-thalassemia. Orphanet J Rare Dis 2010;**5**: 11.*

52. *Hankins J, Hinds P, Day S, Carroll Y, Li CS, Garvie P, Wang W. Therapy preference and decision-making among patients with severe sickle cell anemia and their families. Pediatr Blood Cancer 2007;**48**: 705-10.*

53. *King KE, Ness PM. Treatment of autoimmune hemolytic anemia. Semin Hematol 2005;**42**: 131-6.*

54. *Webb IJ, Anderson KC. Transfusion support in acute leukemias. Semin Oncol 1997;**24**: 141-6.*

55. *Olivieri N. Thalassaemia: clinical management. Baillieres Clin Haematol 1998;**11**: 147-62.*

56. *Pootrakul P, Kitcharoen K, Yansukon P, Wasi P, Fucharoen S, Charoenlarp P, Brittenham G, Pippard MJ, Finch CA. The effect of erythroid hyperplasia on iron balance. Blood 1988;**71**: 1124-9.*

57. *Cohen A, Gayer R, Mizanin J. Long-term effect of splenectomy on transfusion requirements in thalassemia major. Am J Hematol 1989;**30**: 254-6.*

58. *Sirchia G, Zanella A, Parravicini A, Morelati F, Rebulla P, Masera G. Red cell alloantibodies in thalassemia major. Results of an Italian cooperative study. Transfusion 1985;**25**: 110-2.*

59. *Michail-Merianou V, Pamphili-Panousopoulou L, Piperi-Lowes L, Pelegrinis E, Karaklis A. Alloimmunization to red cell antigens in thalassemia: comparative study of usual versus better-match transfusion programmes. Vox Sang 1987;**52**: 95-8.*

60. *Spanos T, Karageorga M, Ladis V, Peristeri J, Hatziliami A, Kattamis C. Red cell alloantibodies in patients with thalassemia. Vox Sang 1990;**58**: 50-5.*

61. *Heddle NM, Soutar RL, O'Hoski PL, Singer J, McBride JA, Ali MA, Kelton JG. A prospective study to determine the frequency and clinical significance of alloimmunization post-transfusion. Br J Haematol 1995;**91**: 1000-5.*

62. *Singer ST, Wu V, Mignacca R, Kuypers FA, Morel P, Vichinsky EP. Alloimmunization and erythrocyte autoimmunization in transfusion-dependent thalassemia patients of predominantly asian descent. Blood 2000;**96**: 3369-73.*

63. *Karimi M, Nikrooz P, Kashef S, Jamalian N, Davatolhagh Z. RBC alloimmunization in blood transfusion-dependent beta-thalassemia patients in southern Iran. Int J Lab Hematol 2007;**29**: 321-6.*

64. *Azarkeivan A, Ansari S, Ahmadi MH, Hajibeigy B, Maghsudlu M, Nasizadeh S, Shaigan M, Toolabi A, Salahmand M. Blood transfusion and alloimmunization in patients with thalassemia: multicenter study. Pediatr Hematol Oncol 2011;**28**: 479-85.*

65. *Saied DA, Kaddah AM, Badr Eldin RM, Mohaseb SS. Alloimmunization and erythrocyte autoimmunization in transfusion-dependent Egyptian thalassemic patients. J Pediatr Hematol Oncol 2011;**33**: 409-14.*

66. *Zimring JC, Welniak L, Semple JW, Ness PM, Slichter SJ, Spitalnik SL. Current problems and future directions of transfusion-induced alloimmunization: summary of an NHLBI working group. Transfusion 2011;**51**: 435-41.*

67. *Petz LD. "Least incompatible" units for transfusion in autoimmune hemolytic anemia: should we eliminate this meaningless term? A commentary for clinicians and transfusion medicine professionals. Transfusion 2003;**43**: 1503-7.*

68. *Higgs DR, Engel JD, Stamatoyannopoulos G. Thalassaemia. Lancet 2011.*

69. *Piomelli S, Danoff SJ, Becker MH, Lipera MJ, Travis SF. Prevention of bone malformations and cardiomegaly in Cooley's anemia by early hypertransfusion regimen. Ann N Y Acad Sci 1969;**165**: 427-36.*

70. *Brittenham GM, Griffith PM, Nienhuis AW, McLaren CE, Young NS, Tucker EE, Allen CJ, Farrell DE, Harris JW. Efficacy of deferoxamine in preventing complications of iron overload in patients with thalassemia major. N Engl J Med 1994;**331**: 567-73.*

71. *Wayne AS, Kevy SV, Nathan DG. Transfusion management of sickle cell disease. Blood 1993;**81**: 1109-23.*

72. *Wanko SO, Telen MJ. Transfusion management in sickle cell disease. Hematol Oncol Clin North Am 2005;**19**: 803-26, v-vi.*

73. *Bunn HF. Pathogenesis and treatment of sickle cell disease. N Engl J Med 1997;**337**: 762-9.*

74. *Powars D, Wilson B, Imbus C, Pegelow C, Allen J. The natural history of stroke in sickle cell disease. Am J Med 1978;**65**: 461-71.*

75. *Adams RJ, McKie VC, Brambilla D, Carl E, Gallagher D, Nichols FT, Roach S, Abboud M, Berman B, Driscoll C, Files B, Hsu L, Hurlet A, Miller S, Olivieri N, Pegelow C, Scher C, Vichinsky E, Wang W, Woods G, Kutlar A, Wright E, Hagner S, Tighe F, Waclawiw MA, et al. Stroke prevention trial in sickle cell anemia. Control Clin Trials 1998;**19**: 110-29.*

76. *Lee MT, Piomelli S, Granger S, Miller ST, Harkness S, Brambilla DJ, Adams RJ. Stroke Prevention Trial in Sickle Cell Anemia (STOP): extended follow-up and final results. Blood 2006;**108**: 847-52.*

77. *Orlina AR, Unger PJ, Koshy M. Post-transfusion alloimmunization in patients with sickle cell disease. Am J Hematol 1978;**5**: 101-6.*

78. *Davies SC, McWilliam AC, Hewitt PE, Devenish A, Brozovic M. Red cell alloimmunization in sickle cell disease. Br J Haematol 1986;**63**: 241-5.*

79. *Ambruso DR, Githens JH, Alcorn R, Dixon DJ, Brown LJ, Vaughn WM, Hays T. Experience with donors matched for minor blood group antigens in patients with sickle cell anemia who are receiving chronic transfusion therapy. Transfusion 1987;**27**: 94-8.*

80. *Cox JV, Steane E, Cunningham G, Frenkel EP. Risk of alloimmunization and delayed hemolytic transfusion reactions in patients with sickle cell disease. Arch Intern Med 1988;**148**: 2485-9.*

81. *Rosse WF, Gallagher D, Kinney TR, Castro O, Dosik H, Moohr J, Wang W, Levy PS. Transfusion and alloimmunization in sickle cell disease. The Cooperative Study of Sickle Cell Disease. Blood 1990;**76**: 1431-7.*

82. *Vichinsky EP, Earles A, Johnson RA, Hoag MS, Williams A, Lubin B. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. N Engl J Med 1990;**322**: 1617-21.*

83. *Sosler SD, Jilly BJ, Saporito C, Koshy M. A simple, practical model for reducing alloimmunization in patients with sickle cell disease. Am J Hematol 1993;**43**: 103-6.*

84. *Tahhan HR, Holbrook CT, Braddy LR, Brewer LD, Christie JD. Antigen-matched donor blood in the transfusion management of patients with sickle cell disease. Transfusion 1994;**34**: 562-9.*

85. *Tchekmedyian NS. Anemia in cancer patients: significance, epidemiology, and current therapy. Oncology (Williston Park) 2002;**16**: 17-24.*

86. *Pisciotta AV. Clinical and laboratory correlation in severe autoimmune hemolytic anemia. AMA Arch Intern Med 1959;**104**: 264-76.*

87. *Petz LD, Fudenberg HH. Coombs-positive hemolytic anemia caused by penicillin administration. N Engl J Med 1966;**274**: 171-8.*

88. *Janeway C. A., Travers P., Walport M., M.J. S, editors. Immunobiology. The Immune system in Health and Disease. 6 ed. New York: Garland Science Publishing, 2005.*

89. *Carstairs KC, Breckenridge A, Dollery CT, Worlledge SM. Incidence of a positive direct coombs test in patients on alpha-methyldopa. Lancet 1966;**2**: 133-5.*

90. *Dacie JV, Cutbush M. Specificity of auto-antibodies in acquired haemolytic anaemia. J Clin Pathol 1954;**7**: 18-21.*

91. *Branch DR, Petz LD. Detecting alloantibodies in patients with autoantibodies. Transfusion 1999;**39**: 6-10.*

92. *Hod EA, Spitalnik SL. Harmful effects of transfusion of older stored red blood cells: iron and inflammation. Transfusion 2011;**51**: 881-5.*

93. *Magnus SA, Hambleton IR, Moosdeen F, Serjeant GR. Recurrent infections in homozygous sickle cell disease. Arch Dis Child 1999;**80**: 537-41.*

94. *Gangaidzo IT, Moyo VM, Mvundura E, Aggrey G, Murphree NL, Khumalo H, Saungweme T, Kasvosve I, Gomo ZA, Rouault T, Boelaert JR, Gordeuk VR. Association of pulmonary tuberculosis with increased dietary iron. J Infect Dis 2001;**184**: 936-9.*

95. *Hod EA, Zhang N, Sokol SA, Wojczyk BS, Francis RO, Ansaldi D, Francis KP, Della-Latta P, Whittier S, Sheth S, Hendrickson JE, Zimring JC, Brittenham GM, Spitalnik SL. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. Blood 2010;**115**: 4284-92.*

96. *Luten M, Roerdinkholder-Stoelwinder B, Schaap NP, de Grip WJ, Bos HJ, Bosman GJ. Survival of red blood cells after transfusion: a comparison between red cells concentrates of different storage periods. Transfusion 2008;**48**: 1478-85.*

97. *Halasz NA, Orloff MJ, Hirose F. Increased Survival of Renal Homografts in Dogs after Injection of Graft Donor Blood. Transplantation 1964;**2**: 453-8.*

98. *Salvatierra O, Jr., Vincenti F, Amend W, Potter D, Iwaki Y, Opelz G, Terasaki P, Duca R, Cochrum K, Hanes D, Stoney RJ, Feduska NJ. Deliberate donor-specific blood transfusions prior to living related renal transplantation. A new approach. Ann Surg 1980;**192**: 543-52.*

99. *Desmarets M, Cadwell CM, Peterson KR, Neades R, Zimring JC. Minor histocompatibility antigens on transfused leukoreduced units of red blood cells induce bone marrow transplant rejection in a mouse model. Blood 2009;**114**: 2315-22.*

100. *Honig CL, Bove JR. Transfusion-associated fatalities: review of Bureau of Biologics reports 1976-1978. Transfusion 1980;**20**: 653-61.*

101. *Mollison PL. Investigation of Haemolytic Transfusion Reactions-II. Br Med J 1943;**1**: 559-61.*

102. *Pineda AA, Taswell HF, Brzica SM, Jr. Transfusion reaction. An immunologic hazard of blood transfusion. Transfusion 1978;**18**: 1-7.*

103. *Schmidt PJ. The mortality from incompatible transfusion. Prog Clin Biol Res 1980;**43**: 251-61.*

104. *Sazama K. Reports of 355 transfusion-associated deaths: 1976 through 1985. Transfusion 1990;**30**: 583-90.*

105. *Nicholls MD. Transfusion: morbidity and mortality. Anaesth Intensive Care 1993;**21**: 15-9.*

106. *Guidance for Industry. Notifying FDA of Fatalities Related to Blood Collection of Transfusion. In: Services DoHaH, ed. Silver Spring: U.S. Food and Drug Administration, 2003.*

107. *Linden JV, Paul B, Dressler KP. A report of 104 transfusion errors in New York State. Transfusion 1992;**32**: 601-6.*

108. *Linden JV, Wagner K, Voytovich AE, Sheehan J. Transfusion errors in New York State: an analysis of 10 years' experience. Transfusion 2000;**40**: 1207-13.*

109. *Fatalities Reported to FDA Following Blood Collection and Transfusion. Annual Summary for Fiscal Year 2010. In: Services DoHaH, ed. Silver Spring: U.S. Foud and Drug Administration, 2010.*

110. *Eklund J, Nevanlinna HR. Rh prevention: a report and analysis of a national programme. J Med Genet 1973;**10**: 1-7.*

111. *Hartmann O, Brendemoen OJ. Incidence of Rh antibody formation in first pregnancies; outcome of pregnancies in 23 cases not previously sensitized to Rh antigens. Acta Paediatr 1953;**42**: 20-3.*

112. *Pollack W, Gorman JG, Freda VJ, Ascari WQ, Allen AE, Baker WJ. Results of clinical trials of RhoGAM in women. Transfusion 1968;**8**: 151-3.*

113. *Antonelou MH, Kriebardis AG, Papassideri IS. Aging and death signalling in mature red cells: from basic science to transfusion practice. Blood Transfus 2010;**8 Suppl 3**: s39-47.*

114. *Ensinck A, Biondi CS, Marini A, Garcia Borras S, Racca LL, Cotorruelo CM, Racca AL. Effect of membrane-bound IgG and desialysation in the interaction of monocytes with senescent erythrocytes. Clin Exp Med 2006;**6**: 138-42.*

115. *Walker WS, Singer JA, Morrison M, Jackson CW. Preferential phagocytosis of in vivo aged murine red blood cells by a macrophage-like cell line. Br J Haematol 1984;**58**: 259-66.*

116. *Kay MM, Wyant T, Goodman J. Autoantibodies to band 3 during aging and disease and aging interventions. Ann N Y Acad Sci 1994;**719**: 419-47.*

117. *Bosman GJ, Stappers M, Novotny VM. Changes in band 3 structure as determinants of erythrocyte integrity during storage and survival after transfusion. Blood Transfus 2010;**8 Suppl 3**: s48-52.*

118. *Kay MM, Bosman GJ, Shapiro SS, Bendich A, Bassel PS. Oxidation as a possible mechanism of cellular aging: vitamin E deficiency causes premature aging and IgG binding to erythrocytes. Proc Natl Acad Sci U S A 1986;**83**: 2463-7.*

119. *Bosman GJ, Werre JM, Willekens FL, Novotny VM. Erythrocyte ageing in vivo and in vitro: structural aspects and implications for transfusion. Transfus Med 2008;**18**: 335-47.*

120. *Arese P, Turrini F, Schwarzer E. Band 3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. Cell Physiol Biochem 2005;**16**: 133-46.*

121. *Willekens FL, Roerdinkholder-Stoelwinder B, Groenen-Dopp YA, Bos HJ, Bosman GJ, van den Bos AG, Verkleij AJ, Werre JM. Hemoglobin loss from erythrocytes in vivo results from spleen-facilitated vesiculation. Blood 2003;**101**: 747-51.*

122. *Connor J, Pak CC, Schroit AJ. Exposure of phosphatidylserine in the outer leaflet of human red blood cells. Relationship to cell density, cell age, and clearance by mononuclear cells. J Biol Chem 1994;**269**: 2399-404.*

123. *Lee SJ, Park SY, Jung MY, Bae SM, Kim IS. Mechanism for phosphatidylserine-dependent erythrophagocytosis in mouse liver. Blood 2011;**117**: 5215-23.*

124. *Willekens FL, Werre JM, Kruijt JK, Roerdinkholder-Stoelwinder B, Groenen-Dopp YA, van den Bos AG, Bosman GJ, van Berkel TJ. Liver Kupffer cells rapidly remove*

*red blood cell-derived vesicles from the circulation by scavenger receptors. Blood 2005;**105**: 2141-5.*

125. *Wolfe LC. The membrane and the lesions of storage in preserved red cells. Transfusion 1985;**25**: 185-203.*

126. *Lang F, Lang KS, Lang PA, Huber SM, Wieder T. Mechanisms and significance of eryptosis. Antioxid Redox Signal 2006;**8**: 1183-92.*

127. *Garratty G, editor Immunobiology of Transfusion Medicine New York: Marcel Dekker, 1994.*

128. *Archer GT. Phagocytosis by human monocytes in red cells coated with Rh antibodies. Vox Sang 1965;**10**: 590-8.*

129. *Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, Lambris JD, Warner RL, Flierl MA, Hoesel LM, Gebhard F, Younger JG, Drouin SM, Wetsel RA, Ward PA. Generation of C5a in the absence of C3: a new complement activation pathway. Nat Med 2006;**12**: 682-7.*

130. *Risitano AM, Notaro R, Marando L, Serio B, Ranaldi D, Seneca E, Ricci P, Alfinito F, Camera A, Gianfaldoni G, Amendola A, Boschetti C, Di Bona E, Fratellanza G, Barbano F, Rodeghiero F, Zanella A, Iori AP, Selleri C, Luzzatto L, Rotoli B. Complement fraction 3 binding on erythrocytes as additional mechanism of disease in paroxysmal nocturnal hemoglobinuria patients treated by eculizumab. Blood 2009;**113**: 4094-100.*

131. *Schreiber AD, Frank MM. Role of antibody and complement in the immune clearance and destruction of erythrocytes. I. In vivo effects of IgG and IgM complement-fixing sites. J Clin Invest 1972;**51**: 575-82.*

132. *Atkinson JP, Frank MM. Studies on the in vivo effects of antibody. Interaction of IgM antibody and complement in the immune clearance and destruction of erythrocytes in man. J Clin Invest 1974;**54**: 339-48.*

133. *Gros P, Milder FJ, Janssen BJ. Complement driven by conformational changes. Nat Rev Immunol 2008;**8**: 48-58.*

134. *Frank MM, Basta M, Fries LF. The effects of intravenous immune globulin on complement-dependent immune damage of cells and tissues. Clin Immunol Immunopathol 1992;**62**: S82-6.*

135. *van Lookeren Campagne M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. Cell Microbiol 2007;**9**: 2095-102.*

136. *Krych-Goldberg M, Atkinson JP. Structure-function relationships of complement receptor type 1. Immunol Rev 2001;**180**: 112-22.*

137. *Fearon DT. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. Proc Natl Acad Sci U S A 1979;**76**: 5867-71.*

138. *Medof ME, Iida K, Mold C, Nussenzweig V. Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes. J Exp Med 1982;**156**: 1739-54.*

139. *Iida K, Nussenzweig V. Complement receptor is an inhibitor of the complement cascade. J Exp Med 1981;**153**: 1138-50.*

140. *Nielsen H, Svehag SR. Detection and differentiation of immune complexes and IgG aggregates by a complement consumption assay. Acta Pathol Microbiol Scand C 1976;**84**: 261-9.*

141. *Muratsugu M. Mechanism of aggregates generated by heating human serum. Biol Pharm Bull 1996;**19**: 132-5.*

142. *Drake WP, Pokorney DR, Mardiney MR, Jr. In vivo abrogation of serum C3 and C5 by administration of cobra venom factor and heterologous anti-C3. J Immunol Methods 1974;**6**: 61-72.*

143. *Gessner JE, Heiken H, Tamm A, Schmidt RE. The IgG Fc receptor family. Ann Hematol 1998;**76**: 231-48.*

144. *Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity 2005;**23**: 41-51.*

145. *Atkinson JP, Schreiber AD, Frank MM. Effects of corticosteroids and splenectomy on the immune clearance and destruction of erythrocytes. J Clin Invest 1973;**52**: 1509-17.*

146. *Jones NC, Mollison PL, Veall N. Removal of incompatible red cells by the spleen. Br J Haematol 1957;**3**: 125-33.*

147. *Lobatto S, Daha MR, Voetman AA, Evers-Schouten JH, Van Es AA, Pauwels EK, Van Es LA. Clearance of soluble aggregates of human immunoglobulin G in healthy volunteers and chimpanzees. Clin Exp Immunol 1987;**69**: 133-41.*

148. *McFadzean AJ, Todd D, Tsang KC. Observations on the anemia of cryptogenetic splenomegaly. I. Hemolysis. Blood 1958;**13**: 513-23.*

149. *Jandl JH, Jones AR, Castle WB. The destruction of red cells by antibodies in man. I. Observations of the sequestration and lysis of red cells altered by immune mechanisms. J Clin Invest 1957;**36**: 1428-59.*

150. *Mollison PL, Hughes Jones NC. Sites of removal of incompatible red cells from the circulation. Vox Sang 1958;**3**: 243-51.*

151. *Brown DL, Lachmann PJ, Dacie JV. The in vivo behaviour of complement-coated red cells: studies in C6-deficient, C3-depleted and normal rabbits. Clin Exp Immunol 1970;**7**: 401-21.*

152. *Cutbush M, Mollison PL. Relation between characteristics of blood-group antibodies in vitro and associated patterns of redcell destruction in vivo. Br J Haematol 1958;**4**: 115-37.*

153. *Mollison PL. The reticulo-endothelial system and red cell destruction. Proc R Soc Med 1962;**55**: 915-20.*

154. *Mollison PL, Hughes-Jones NC. Clearance of Rh-positive red cells by low concentrations of Rh antibody. Immunology 1967;**12**: 63-73.*

155. *Mollison PL. Blood-group antibodies and red-cell destruction. Br Med J 1959;**2**: 1123-30.*

156. *Mollison PL, Johnson CA, Prior DM. Dose-dependent destruction of A1 cells by anti-A1. Vox Sang 1978;**35**: 149-53.*

157. *Brain MC, Pihl C, Robertson L, Brown CB. Evidence for a mechanosensitive calcium influx into red cells. Blood Cells Mol Dis 2004;**32**: 349-52.*

158. *Brain MC, Ruether B, Valentine K, Brown C, ter Keurs H. Life-threatening hemolytic anemia due to an autoanti-Pr cold agglutinin: evidence that glycophorin A antibodies may induce lipid bilayer exposure and cation permeability independent of agglutination. Transfusion 2010;**50**: 292-301.*

159. *Garratty G. A new mechanism for immune destruction of red blood cells? Transfusion 2010;**50**: 274-7.*

160. *Attanasio P, Shumilina E, Hermle T, Kiedaisch V, Lang PA, Huber SM, Wieder T, Lang F. Stimulation of eryptosis by anti-A IgG antibodies. Cell Physiol Biochem 2007;**20**: 591-600.*

161. *Shibata T, Berney T, Reininger L, Chicheportiche Y, Ozaki S, Shirai T, Izui S. Monoclonal anti-erythrocyte autoantibodies derived from NZB mice cause autoimmune hemolytic anemia by two distinct pathogenic mechanisms. Int Immunol 1990;**2**: 1133-41.*

162. *van der Meulen FW, de Bruin HG, Goosen PC, Bruynes EC, Joustra-Maas CJ, Telkamp HG, von dem Borne AE, Engelfriet CP. Quantitative aspects of the destruction of red cells sensitized with IgG1 autoantibodies: an application of flow cytofluorometry. Br J Haematol 1980;**46**: 47-56.*

163. *Garratty G, Nance SJ. Correlation between in vivo hemolysis and the amount of red cell-bound IgG measured by flow cytometry. Transfusion 1990;**30**: 617-21.*

164. *Hod EA, Zimring JC, Spitalnik SL. Lessons learned from mouse models of hemolytic transfusion reactions. Curr Opin Hematol 2008;**15**: 601-5.*

165. *Hod EA, Arinsburg SA, Francis RO, Hendrickson JE, Zimring JC, Spitalnik SL. Use of mouse models to study the mechanisms and consequences of RBC clearance. Vox Sang 2010;**99**: 99-111.*

166. *Schirmer DA, Song SC, Baliff JP, Harbers SO, Clynes RA, Krop-Watorek A, Halverson GR, Czerwinski M, Spitalnik SL. Mouse models of IgG- and IgM-mediated hemolysis. Blood 2007;**109**: 3099-107.*

167. *Mollison PL. Factors determining the relative clinical importance of different blood-group antibodies. Br Med Bull 1959;**15**: 92-8.*

168. *Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Role of CD47 as a marker of self on red blood cells. Science 2000;**288**: 2051-4.*

169. *Chaplin H, Nasongkla M, Monroe MC. Quantitation of red blood cell-bound C3d in normal subjects and random hospitalized patients. Br J Haematol 1981;**48**: 69-78.*

170. *Merry AH, Thomson EE, Rawlinson VI, Stratton F. The quantification of C3 fragments on erythrocytes: estimation of C3 fragments on normal cells, acquired haemolytic anaemia cases and correlation with agglutination of sensitized cells. Clin Lab Haematol 1983;**5**: 387-97.*

171. *Rosenfield RE, Jagathambal. Antigenic determinants of C3 and C4 complement components on washed erythrocytes from normal persons. Transfusion 1978;**18**: 517-23.*

172. *Merry AH, Thomson EE, Rawlinson VI, Stratton F. A quantitative antiglobulin test for IgG for use in blood transfusion serology. Clin Lab Haematol 1982;**4**: 393-402.*

173. *Jeje MO, Blajchman MA, Steeves K, Horsewood P, Kelton JG. Quantitation of red cell-associated IgG using an immunoradiometric assay. Transfusion 1984;**24**: 473-6.*

174. *Moller G. Survival of Mouse Erythrocytes in Histoincompatible Recipients. Nature 1963;**199**: 573-5.*

175. *Moller G. Isoantibody-Induced Cellular Resistance to Immune Haemolysis in Vivo and in Vitro. Nature 1964;**202**: 357-9.*

176. *Zimring JC, Cadwell CM, Spitalnik SL. Antigen loss from antibody-coated red blood cells. Transfus Med Rev 2009;**23**: 189-204.*

177. *Zimring JC, Hair GA, Chadwick TE, Deshpande SS, Anderson KM, Hillyer CD, Roback JD. Nonhemolytic antibody-induced loss of erythrocyte surface antigen. Blood 2005;**106**: 1105-12.*

178. *Zimring JC, Cadwell CM, Chadwick TE, Spitalnik SL, Schirmer DA, Wu T, Parkos CA, Hillyer CD. Nonhemolytic antigen loss from red blood cells requires cooperative binding of multiple antibodies recognizing different epitopes. Blood 2007;**110**: 2201-8.*

179. *Mollison PL, Cutbush M. The use of isotope-labelled red cells to study incompatibility. Bibl Haematol 1958;**7**: 50-4.*

180. *Mollison PL, Cutbush M. Use of isotope-labelled red cells to demonstrate incompatibility in vivo. Lancet 1955;**268**: 1290-5.*

Chapter 2

Biphasic clearance of incompatible RBCs through a novel mechanism requiring neither complement nor Fc gamma receptors in a murine model

Biphasic clearance of incompatible RBCs through a novel mechanism requiring neither complement nor Fc gamma receptors in a murine model

Justine S. Liepkalns[1], Eldad A. Hod[2], Sean R. Stowell[1], Chantel M. Cadwell[1], Steven L. Spitalnik[2], James C. Zimring[1,3*]

1. Center for Transfusion and Cellular Therapies, Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia 30322.

2. Department of Pathology and Cell Biology, Columbia University College of Physicians and Surgeons - New York Presbyterian Hospital, New York, NY, 10032

3. Aflac Cancer Center, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, 30322

* To whom all correspondence should be addressed

Please address correspondence to:

James C. Zimring, M.D., Ph.D., Emory University School of Medicine, Woodruff Memorial Building Suite 7107, 101 Woodruff Circle, Atlanta, GA 30322, USA (Telephone 404-712-2174, Fax 404-727-5764) Email jzimrin@emory.edu

Word count
Abstract = 239
Body = 4979
Tables =0
Figures =7
References=47

Conflicts of Interest: The authors have no conflicts of interest to declare

Running Title: Novel hemolytic transfusion reaction

Abstract

Antibody binding to red blood cells (RBCs) can induce potentially fatal outcomes, including hemolytic transfusion reactions (HTRs), hemolytic disease of the fetus and newborn and autoimmune hemolytic anemia. The mechanism(s) of RBC destruction following antibody binding is typically thought to require complement activation and/or the involvement of Fc gamma receptors (FcγRs). In the current report, we analyzed mechanisms of HTRs during incompatible transfusions of murine RBCs expressing human Glycophorin A (hGPA) into mice with anti-hGPA antibodies. C3 and Fcγ receptor knockout, splenectomized, Fcγ receptor blocking antibody treated and clodronate treated mice were passively immunized with anti-hGPA antibodies (10F7 or 6A7) and transfused with RBCs expressing the hGPA antigen. Post transfusion blood and serum were collected and analyzed via flow cytometry and confocal microscopy. This HTR model results in both rapid clearance and cytokine storm. Neither complement nor FcγRs were required for RBC clearance; in contrast, FcγRs were required for cytokine storm. Circulating aggregates of hGPA RBCs were visible during the HTR. Splenectomy and phagocyte depletion by clodronate had no effect on acute RBC clearance; however incompatible RBCs re-entered over 24 hours in clodronate treated mice. These data demonstrate a biphasic HTR; the first phase involving sequestration of incompatible hGPA RBCs and the second phase involving phagocytosis of sequestered RBCs. However, the mechanism(s) of phagocytosis in the second phase did require neither C3 nor FcγRs. These findings demonstrate novel mechanistic biology of HTRs.

Introduction:

Crossmatch incompatible transfusions consist of infusing donor red blood cells (RBCs) into a recipient who has antibodies against antigens on the donor RBCs. Except for naturally occurring blood group antibodies (e.g. the ABO system), alloantibodies to RBC antigens are generated through prior exposure to allogeneic RBCs, typically by transfusion or pregnancy.[1] In general, incompatible transfusions are strictly avoided, as hemolysis of the transfused RBCs can occur with potentially fatal outcomes, known as hemolytic transfusion reactions (HTRs).[2,3] HTRs can occur during primary alloimmunization, as antibodies can be generated in 12-14 days, whereas transfused RBCs have a life span of up to several months. Additional HTRs result from clerical error and mistransfusion.[4,5] Moreover, in other instances, crossmatch incompatible RBCs may be purposefully transfused if the immediate risks of hypoxia (e.g. due to anemia) outweigh the potential damage from an induced HTR. Alloantibodies against fetal RBCs can also cause hemolytic disease of the fetus and newborn (HDFN), resulting in substantial morbidity and/or mortality.[6] Finally, autoantibodies bound to RBCs can cause autoimmune hemolytic anemia (AIHA).[7] Thus, an understanding of the mechanisms by which antibody bound RBCs are cleared from the circulation and the pathophysiology that ensues is important for several disease settings.

Antibody induced hemolysis is generally thought to occur by one of two mechanisms.[8] First, RBCs can undergo intravascular hemolysis when complement is activated to form the membrane attack complex (MAC). This is typically due to IgM binding to the RBC surface, but also occasionally occurs with IgG.[9,10] RBCs can also be opsonized and ingested by phagocytes, a process referred to as extravascular hemolysis. If complement activation does not lead to MAC formation to the extent that rapid intravascular hemolysis occurs, then C3 deposited on the RBC surface may be

converted to C3b and iC3b. In this case, complement receptors on phagocytes (i.e. CR1,CR2, and CR3) can consume C3b-coated RBCs. Second, antibody induced opsonization also occurs due to Fc domains of IgG bound to RBCs, which are recognized by Fc gamma receptors (FcγRs) on phagocytes, mostly extravascularly. There are substantial and significant data, generated mostly in animal models that demonstrate the existence of each of these pathways of RBC clearance after antibody binding.[8,11]

In contrast to the canonical pathways described above, alternate mechanisms of IgG-mediated RBC clearance have been suggested, which involve neither complement nor FcγR pathways. These include direct effects upon the RBC by antibody binding. Destabilization of the RBC membrane can induce RBC death (eryptosis).[12,13] Antibodies have also been shown to directly induce phosphatidylserine expression on the RBC surface, as an indication for phagocytosis.[12] Moreover, mechanical induction of Ca^{2+} influx into RBCs has also been demonstrated as a direct effect of antibody surface binding.[14,15] These pathways are not mutually exclusive and may overlap. Although these alternate pathways raise important mechanistic questions regarding HTRs, they have been identified *in vitro*; therefore, it is unclear if these findings are relevant to authentic HTRs *in vivo*. Finally, RBC removal from circulation *in vivo* by sequestration in the spleen and liver has been observed in the context of IgG and IgA autoimmune hemolytic anemia, but a role for sequestration has not been reported in the context of incompatible transfusion.[16,17]

Herein, we utilize an *in vivo* murine model of incompatible RBC transfusion involving IgG antibodies in the recipient against human glycophorin A (hGPA) epitopes on donor RBCs. The 6A7 and 10F7 mouse monoclonal antibodies recognize the antithetical M human blood group antigen or a non-polymorphic common hGPA epitope,

respectively.[18-20] Our data demonstrate clearance of RBCs by a mechanism requiring neither complement nor FcγRs. To our knowledge, this is the first report identifying this alternate pathway of IgG antibody-induced hemolysis *in vivo* in the setting of crossmatch incompatible transfusion. In addition, our findings mechanistically dissociate RBC clearance from phagocyte ingestion and cytokine storm. Together, these findings provide insights into a novel HTR mechanism in mice, the prevalence of which is unknown in human HTRs.

Methods:

Mice

C57BL/6 (B6), FVB,[21] and C3 knockout mice were purchased from the Jackson Laboratories (Bar Harbor, ME). FVB mice are advantageous in transgenic work due to their large pronucleus, facilitating DNA microinjections, and to their large litters.[21] FcγR KO mice (Fcer1g) were purchased from Taconic Farms, Inc. All mice were used at 8-16 weeks of age. Transgenic mice expressing the M variant of the human Glycophorin A (hGPA) on RBCs were obtained from the New York Blood Center.[22] HOD mice consisted of transgenic animals expressing a fusion protein Hen Egg Lysozyme (HEL), Ovalbumin (Ova), and Duffy b.[23] Both hGPA and HOD mice are on an FVB background. HOD and hGPA mice were crossed to generate HOD x hGPA F1 mice that express both transgenes. All breeding (including FcγR KO and C3 KO mice) was performed by the Emory University Department of Animal Resources Husbandry Services and all procedures were performed according to approved IACUC protocols.

Antibodies and passive immunization

Anti-hGPA (6A7 and 10F7) mouse monoclonal antibodies are of the IgG1 subtype; 6A7 is specific for the M variant of hGPA[18-20] whereas 10F7 recognizes both M and N variants.[18,19] Anti-Fy^3 (MIMA29) is an IgG2a antibody that binds to the Duffy portion of the HOD antigen on the third extracellular loop (Fy^3) [24] and was a generous gift from Marion Reid and Greg Halverson at the New York Blood Center. In some experiments, 2.4G2, an Fcγ receptor blocking antibody,[25] was administered to recipients i.p. 24 hours prior to passive immunization with anti-hGPA. All above antibodies were purified by protein G chromatography (Bio X Cell, West Lebanon, NH). Recipients were passively immunized by tail vein injection with 500 μg 10F7, 25 μg 6A7, or 200 μg MIMA29 in a

total volume of 500 μL of phosphate buffered saline (PBS), 2-3 hours prior to transfusion; control mice received 500 μL of PBS alone. 10F7 was used at a concentration optimized in a previous study by Schirmer and colleagues[26] doses of 6A7 and MIMA29 were optimized based upon potency in inducing RBC clearance.

Fluorescent labeling, Transfusion, and survival monitoring of murine RBCs

RBC fluorescent labeling, treatment with phenylhydrazine, transfusion, survival determination, and splenectomies were performed as previously described.[27-29] hGPA x HOD, hGPA or HOD RBCs were labeled with 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) and control (FVB) blood was labeled with 3,3'-dihexadecyloxacarbocyanine perchlorate (DiO). The survival of DiI-labeled RBCs was calculated by normalizing it to DiO-labeled wild type FVB RBCs within the same animal. These resulting ratios from passively immunized mice were then normalized to those of PBS-treated animals and graphed as percent hGPA x HOD, hGPA or HOD RBC survival.

Fluorescent Microscopy

Blood samples were collected post transfusion and smears were prepared on glass slides. Smears were air dried overnight to increase RBC adhesion to the slide (in the dark to minimize photobleaching). Slides were then mounted with Vecta Mount (Vector laboratories) and visualized with a point scanning laser confocal microscope (Zeiss LSM 510, Thornwood, NY).

Clodronate treatment of recipient mice

C57BL/6 mice were treated with plain (empty) liposomes (9.4 mg/ml L-α-Phosphatidylcholine, 2.1 mg/ml cholesterol) or liposomes filled with clodronate

(dichloromethylene-bisphosphonate (Cl_2MBP)) intravenously 24 hours prior to passive immunization and transfusion. Each mouse received 1.5 mg of Clodronate in 600 μl total volume. Control mice received the total 600 μl volume in one setting, whereas the experimental mice received clodronate-filled liposomes slowly (about 10μl/second, in two doses of 300 μl each).

Cytokine measurements

Plasma Interleukin (IL)-6, tumor necrosis factor (TNF)-α, monocyte chemo attractant protein (MCP)-1, keratinocyte-derived chemokine (KC), and IL-10 were quantified using the Cytometric Bead Array Mouse Flex Kit (BD Biosciences, San Diego, CA).

Statistical analysis

Survival graphs were all calculated and created with Prism software. All error bars represent one standard deviation. P values are calculated, via log transformation, using a two way ANOVA with Bonferroni post-test using Prism software.

Results:

RBC clearance during crossmatch incompatible transfusion for the M and Fy^3 blood group antigens

hGPA mouse RBCs express transgenic human glycophorin A and HOD mouse RBCs express a triple fusion transgenic protein consisting of Hen Egg Lysozyme, Ovalbumin, and human Duffy. Both hGPA and HOD mice express their respective transgenes in an RBC specific fashion.[22,23] To generate RBC donors with multiple known RBC antigens, hGPA and HOD mice were crossed to yield F1 progeny (hGPA x HOD mice) expressing both the hGPA and HOD transgenes on RBCs (see Figure 2.1A for depiction and antibody binding sites). In some cases the single transgenic parental strains, hGPA or HOD, were used as donors (as indicated by single antigen nomenclature). C57BL/6 recipients were passively immunized with monoclonal anti-hGPA (10F7 or 6A7), or monoclonal anti-Fy^3 (MIMA29), followed by transfusion with a mixture of wild-type FVB RBCs (labeled with DiO) and hGPA x HOD RBCs (labeled with DiI). hGPA x HOD mice are on an FVB background. Analysis of peripheral blood from control animals that were transfused, but not passively immunized, demonstrated that the hGPA (10F7 and 6A7), and Fy^3 epitopes are expressed and detectable on hGPA x HOD, but not on co-transfused control wild-type RBCs (Figure 2.1A).

Survival of transfused RBCs was determined by enumerating labeled RBCs by flow cytometry and normalizing the circulating incompatible RBCs as a function of wild-type RBCs within the same recipient (representative flow plots shown in Figure 2.1B). Both anti-hGPA antibodies (10F7 and 6A7) induced rapid clearance of hGPA x HOD RBCs, with approximately 80% of the RBCs becoming undetectable in peripheral blood by 2 hours post-transfusion; only small amounts of additional clearance were observed

after the 2 hour time point (Figure 2.1C). Anti-Fy^3 induced a similar final degree of clearance, but with slower kinetics (Figure 2.1C). Together, these data define a model of crossmatch incompatible transfusion using 2 distinct antigens on the same donor RBCs and 3 monoclonal antibodies recognizing different epitopes.

Role of complement and FcγRs in clearance of incompatible RBCs

Upon activation of the complement pathway by antibody binding, C3 becomes covalently attached to both the inciting antibodies and the surrounding cellular proteins via a thioester bond.[30] In this process, C3 is converted to C3b and iC3b, which are ligands for complement receptors CR1, CR3 and CR4, which then promote phagocytosis.[8] In addition, C3 is generally required for the complement cascade to proceed to complete MAC assembly.[31] To assess the role of complement in clearance of incompatible RBCs, we utilized recipients with a deletion of the C3 gene (C3 KO mice). Antibody-mediated clearance of transfused hGPA x HOD RBCs was unaltered in C3 KO mice, compared to wild type mice for 10F7 (Figure 2.2A), 6A7 (Figure 2.2B) and anti-Fy^3 (Figure 2.2C). The phenotype of the C3 KO mice was confirmed via a complement fixation assay; normal C3 was present in C57BL/6 mice and was undetectable in C3 KO animals (data not shown). These data demonstrate that C3 is not required for clearance of incompatible RBCs in this model.

To examine the role of FcγRs in clearance of incompatible RBCs, we used mice with a deletion of the common γ chain, which is required for expression and function of the three murine FcγRs known to participate in phagocytosis (FcγRI, FcγRIII, and FcγRIV).[32,33] Only a small decrease in clearance was observed in FcγR KO mice passively immunized with 10F7 or 6A7 as compared to wild type mice (Figure 2.2D and E). In contrast, clearance of hGPA x HOD RBCs induced by anti-Fy^3 was abrogated in FcγR KO mice (Figure 2.2F). These data indicate that FcγRs are required for clearance

of incompatible RBCs by anti-Fy^3, but has only a small effect on clearance by anti-hGPA antibodies.

The above data demonstrate that neither C3 nor FcγRs are individually required for RBC clearance by anti-hGPA antibodies; however, this does not address the possibility that C3 and FcγRs represent redundant pathways, both mediating hGPA RBC clearance. To test for this redundancy, we used monoclonal antibody 2.4G2, which has been reported to block both FcγRII and FcγRIII by binding a conserved epitope.[25,34,35] In wild type recipients, injection of purified 2.4G2 had no appreciable effect on clearance of hGPA x HOD RBCs by 10F7; however, a subtle but significant difference was observed with 6A7 induced clearance (Figure 2.3A). As predicted, based on results with FcγR KO mice, 2.4G2 significantly inhibited clearance of hGPA x HOD RBCs by anti-Fy^3 at 2 hours post-transfusion (Figure 2.3A). This blocking effect was progressively lost over time (see below), which may result from incomplete blockage and/or gradual clearance of the 2.4G2 antibody. Nevertheless, these data confirm that 2.4G2 functionally inhibits clearance of RBCs by an FcγR dependent antibody (anti-Fy^3) at early time points; therefore, it is a useful tool for assessing the FcγR dependence of RBC clearance in this model.

To assess the possibility that C3 and FcγRs are redundant pathways in anti-hGPA mediated clearance, C3 KO mice were treated with 2.4G2 to inhibit both pathways simultaneously. These mice were then passively immunized with anti-hGPA (10F7 or 6A7) or anti-Fy^3 and subsequently transfused. Antibody 2.4G2 had a small effect on clearance by 6A7 (similar to the FcγR KO recipients) and no effect on clearance by 10F7 (Figures 2.3B & 2.3C). Similar to wild-type recipients, 2.4G2 inhibited clearance by anti-Fy^3 at early time points, but this effect diminished over 48 hours (Figure 2.3D). Based

upon these data, we reject the hypothesis that C3 and FcγRs represent redundant pathways in this model and conclude that neither C3 nor FcγRs are required for clearance by these anti-hGPA antibodies.

Phagocytic cells are not required for early clearance of hGPA and HOD RBCs but play a role in preventing return to circulation

Phagocytes are known to use multiple scavenger receptors (in addition to Fc and complement receptors) to remove damaged cells.[36] Therefore, we tested the hypothesis that phagocytes are required for IgG-coated RBC clearance by treating C57BL/6 recipients with clodronate (a toxic electron transport chain decoupling biphosphonate). Clodronate was targeted to phagocytes by intravenous injection of clodronate encapsulated in liposomes, which are consumed by phagocytic cells.[37,38] Control mice were injected with liposomes of the same composition but without clodronate (i.e. empty or "plain" liposomes). Mice were treated 24 hours prior to passive immunization and transfused with a mixture of transgenic (hGPA or HOD) and control wild-type RBCs. In recipients immunized with anti-hGPA (10F7 or 6A7), hGPA RBCs were cleared to the same extent in clodronate and plain liposome-treated animals at the first time point (2 hours post transfusion) (Figure 2.4A and 2.4B respectively). However, starting at 18 hours, most incompatible hGPA RBCs returned to circulation, leading to over 50% survival of incompatible RBCs at 2 days post transfusion (Figure 2.4A and 2.4B). In mice passively immunized with anti-Fy^3, liposomal clodronate infusion prevented initial HOD RBC clearance, confirming that clodronate was effective and resulted in sufficient phagocyte depletion to prevent FcγR dependent RBC clearance.

Histological analysis of cross-sections of spleens from clodronate treated animals demonstrated a large portion of splenic architecture had been compromised (data not shown). We therefore could not distinguish between the interpretations that the

increased survival of incompatible hGPA RBCs was due to phagocyte depletion or to reduced splenic blood filtration. Therefore, we tested the hypothesis that the spleen plays a central role in incompatible hGPA x HOD RBC clearance. To this end, recipient mice were splenectomized prior to passive immunization and transfusion with hGPA x HOD and wild type FVB RBCs. Control mice underwent sham surgery. Incompatible RBCs were cleared with a similar magnitude and kinetics in both sham operated and splenectomized mice when passively immunized with 10F7 (Figure 2.4D), 6A7 (Figure 2.4E) or anti-Fy^3 (Figure 2.4F). These data do not rule out that the spleen is involved; however, these findings do reject the hypothesis that a spleen is required for either anti-hGPA or anti-Fy^3 mediated clearance of incompatible RBCs.

To investigate the persistence of antibody binding to incompatible RBCs that continue to circulate, peripheral blood was stained with an anti-mouse globulin reagent (Direct Antiglobulin Test (DAT)) and analyzed by flow cytometry. In both 10F7 and 6A7 treated animals, DAT levels decreased over time in mice treated with clodronate. In contrast, antibody binding was stable in mice treated with plain liposomes (Figure 2.4G). Antigen persistence was determined by staining RBCs with 10F7 or 6A7, followed by anti-mouse globulin. In contrast to DATs, there was only a subtle decrease in antigen levels over time (Figure 2.4H). It is important to note that in the presence of clodronate, the majority of RBCs at late time points have been sequestered and then returned to circulation. In this context, these data suggest that the RBCs that return to circulation have done so in part by breaking free of the bound antibodies, without lysing or losing their surface antigen. Also of interest, the few RBCs that survive in passively immunized control animals are not escaping clearance due to low levels of antibody binding, but due to some other property. Together, these data support a scenario in which phagocytes are not required for initial RBC clearance, but play an essential role in removing

sequestered RBCs, which otherwise return to circulation in the absence of sufficient phagocytic activity.

Anti-hGPA induces aggregation of transfused incompatible RBCs in vivo

To test the hypothesis that aggregation of RBCs *in vivo* is responsible for anti-hGPA mediated clearance, incompatible transfusions were performed and peripheral blood was analyzed during the rapid clearance phase (i.e. at time points prior to 2 hours post transfusion). To characterize and enumerate agglutination, differential size analysis was performed by analysis of light scatter by flow cytometry. Scatter plots identified a population with increased size and complexity, which was only present under conditions of hGPA x HOD RBC transfusion into mice passively immunized with anti-hGPA, but was absent in both PBS treated controls and anti-Fy^3 injected animals (Figure 2.5A). Gating on the large complexes by forward and side scatter and then back reflection on the entire population (side scatter by DiI staining) demonstrated a substantial increase in the DiI signal (Figure 2.5B and 2.5C) but not the DiO signal (data not shown). This finding indicated that the large aggregates were composed of hGPA x HOD RBCs, but not control RBCs. These data are consistent with the interpretation that the large complexes constitute selective agglutination *in vivo* of incompatible RBCs. These larger complexes were also enumerated over time and correlated with clearance; the complexes were detectable early in the reaction, rapidly decreased during the clearance phase, and ceased to be detectable soon after transfusion (Figure 2.5D). The large complexes were not an artifact of spontaneous aggregation, as no significant complexes were observed in either control mice receiving PBS (i.e. compatible transfusion) or in the clearance of RBCs by anti-Fy^3 (i.e. an FcγR-dependent HTR) (Figure 2.5E).

To visualize incompatible RBCs directly during clearance, the same early time point specimens analyzed above were smeared on microscope slides and analyzed by

confocal microscopy using filters that selectively visualize the emission spectra of DiI (hGPA x HOD RBCs) and DiO (wild type FVB RBCs). All slides were interpreted by viewers blinded to sample identity. DiI^+ RBC clusters were visible in recipients that had been passively immunized with anti-hGPA antibody (either 10F7 or 6A7), but not in unimmunized controls or animals passively immunized with anti-Fy^3 (Figure 2.5F). In contrast, no clusters of DiO^+ RBCs (wild type) were seen in anti-hGPA, anti-Fy^3 or control unimmunized mice (Figure 2.5F). Together, these data show that the presence of DiI-labeled hGPA x HOD RBC clusters (by flow cytometry or microscopy) correlated with the clearance kinetics of hGPA x HOD RBCs by anti-hGPA antibodies (10F7 or 6A7) but not with their anti-Fy^3 treated counterparts.

Increased inflammatory cytokine secretion in C57BL/6 mice, but not in FcγR KO animals during incompatible transfusion with hGPA or HOD RBCs

Phagocytes not only remove pathogens from circulation but also secrete inflammatory cytokines, which can be triggered by FcγR cross-linking by antibody coated RBCs.[39,40] We previously reported that incompatible transfusion of hGPA RBCs into mice passively immunized with 10F7 or 6A7 results in a cytokine burst not seen during compatible transfusion.[40] The data above indicate that FcγRs are not required for clearance of hGPA RBCs. To test the role of FcγRs in cytokine burst, serum cytokines were measured in C57BL/6 and FcγR KO mice 2 hours after incompatible transfusion, during which a robust response has been shown to occur in C57BL/6 mice.[40] Clearance at 2 hours post-transfusion was similar to that previously observed (data not shown). Sera from C57BL/6 mice undergoing hGPA incompatible transfusion (i.e. passively immunized with 10F7 or 6A7) had significantly elevated levels of inflammatory cytokines (IL-6, TNFα, MCP-1 and KC) compared to compatible transfusion (mice infused with PBS) (Figure 2.6A). Likewise, incompatible transfusions of HOD RBCs induced a

cytokine burst in C57BL/6 mice (Figure 2.6B). In contrast to wild-type animals, no significant difference in cytokines was seen in FcγR KO mice receiving incompatible, as compared to compatible, transfusions, in either the hGPA or HOD systems (Figures 2.6A & 2.6B). IL10 levels were also assessed however no changes were observed between each of the groups (data not shown). To assess if the targeted deletion of FcγRs resulted in a phenotype incapable of cytokine burst, FcγR mice were transfused with phenylhydrazine treated RBCs, which we previously reported induces cytokine storm in wild-type mice.[28] A cytokine burst was observed in the FcγR KO mice transfused with phenylhydrazine treated RBCs (although it was substantially lower in magnitude as compared to wild-type mice). Thus, although blunted, FcγR KO mice are capable of releasing a cytokine burst in response to damaged RBCs, indicating that the lack of cytokine burst in response to incompatible transfusion was not due an inability to release cytokines. Taken together, these results indicate that FcγRs are required for cytokine burst 2 hours post-transfusion with incompatible hGPA RBCs, even though FcγRs are not required for either rapid or sustained clearance of RBCs expressing the hGPA antigen.

Discussion

RBC transfusions are typically performed only with blood that is compatible for clinically significant antibodies. However, when mistransfusions occur, or when no other option is available, incompatible RBCs may be infused, which puts the patient at risk of experiencing an HTR. Moreover, antibody-bound RBCs can be cleared from circulation during an amnestic response. Currently, there are no evidence based therapeutic interventions available to prevent the clinical consequences of HTRs after incompatible transfusion. Understanding the mechanisms underlying HTRs is essential for providing a rationale for such interventions.

Canonically, mechanisms explaining IgG-mediated HTRs are divided into two types, involving either complement or FcγRs. In contrast, the current study reveals that for hGPA-encoded antigens, clearance of incompatible RBCs requires neither C3 nor FcγRs. Moreover, as the simultaneous absence of both C3 and FcγR function has no effect on clearance, it is unlikely that C3 and FcγRs represent redundant pathways. Rather, these data suggest that, in this setting, C3 and FcγRs are neither required nor involved. It is worth noting that 2.4G2 does not block FcγRIV function; thus, we cannot unequivocally rule out redundancy between C3 and FcγRIV; nonetheless, because FcγR KO mice lack functional FcγRI, FcγRIII and FcγRIV, we can conclude that FcγRIV is not required for clearance. It is also worth noting that the lack of requirement for C3 does not unequivocally reject a role for complement in general, because C5 can be activated by thrombin in the absence of C3.[41] Thus, it remains theoretically possible that the MAC could still form in some cases; however, clearance of hGPA RBCs by 10F7 and 6A7 occurs normally in DBA.2 (data not shown) and FVB mice,[26] both of which are deficient in C5. Perhaps more importantly, RBCs lysed by the MAC would not be able to return to circulation in the absence of clodronate sensitive phagocytes, as MAC assembly results

in RBC lysis. A mechanism of sequestration has previously been proposed involving complement receptor binding to RBC-bound C3b, which is cleaved and the RBC is subsequently released back into circulation. CR1 has been shown to preferentially bind C4b and C3b.[42,43] Once bound to CR1, proteolytic cleavage of C3b can be enhanced.[44,45] Since CR1 is found mostly on phagocytes, we partially address its involvement in sequestration during clodronate treatment. We cannot, however rule out that CR1 is required for sequestration because of its known presence on RBCs.[43] Although we demonstrated no diminished clearance of incompatible hGPA RBCs even at early time points in C3KO animals, we cannot rule out that initial sequestration involves to CR1 interactions with surface bound C4b.

Taken together, the data presented herein allow the conclusion that hGPA RBCs are cleared by IgG alloantibodies using a mechanism(s) independent of C3 or FcγRs. The existence of such pathways for IgG mediated HTRs has been postulated by others,[12,14,15,17,46] but to our knowledge, has not been demonstrated *in vivo*. Agglutination *in vivo* was observed in a murine model of AIHA induced by IgG monoclonal antibodies, although every circulating RBC was antigen positive in this case, in contrast to incompatible transfusions, where antigen positive RBCs are more dispersed.[17] In the current model, the visualization of aggregate formation by flow cytometry and confocal microscopy suggests that agglutination *in vivo* is a likely mechanism of initial clearance. Aggregation is not an inevitable outcome of murine HTR models, because agglutination did not correlate with incompatible RBC clearance by an anti-Fy^3 antibody. Further study of different blood group systems will be required to determine if this mechanism is found in additional settings or is restricted to HTRs in the hGPA system. The distinct mechanism by hGPA and HOD RBCs may be due to differences in the number of copies expressed, the IgG subtype used during passive immunization, the nature of the membrane protein and/or protein associations.

It is unclear to what extent these findings predict mechanisms of HTRs in humans. Agglutination by IgM and IgG antibodies has been observed in AIHA patients. Moreover, sequestration of incompatible cells outside of the circulation has been observed in human studies of transfused radioactively labeled RBCs[47-49] including a release of a small number of sequestered incompatible RBCs.[47] However, since the human studies were performed using radioactivity it is difficult to distinguish between sequestration of intact RBCs versus consumption of radiolabeled RBCs. It is likewise difficult to distinguish between release of previous sequestered RBCs versus circulation of phagocytes that have consumed radiolabeled RBCs. Use of flow cytometry in the current studies avoids these ambiguities.

Several important characteristics can be concluded from the current studies. First, as splenectomy does not affect clearance, removal of the incompatible RBCs can occur in extrasplenic sites. The spleen is therefore not required but may be involved in clearance of incompatible RBCs. Second, although it is not clear whether sensitivity to clodronate varies with different organs, clodronate treatment results in a return of incompatible RBCs to the circulation over time. This observation suggests that phagocytes are required to prevent initially cleared RBCs from reentering the vasculature, presumably by ingesting the antibody bound RBCs. However, as no return to the circulation is seen in C3 KO or FcγR KO mice, the signal for ingestion appears to be something other than opsonization with complement or IgG. The identity of this additional signal is unclear from the current data; however, Brain *et al.* showed that binding of polyclonal human IgG antibodies (from a patient with a HTR due to anti-Pr) can directly damage RBCs, including membrane distortions, opening of Ca^{2+} channels and exposure of phosphatidylethanolamine.[15] Thus, it is possible that 10F7 and 6A7 induce expression of an “eat me signal”, such as phosphatidylserine, which is then recognized by scavenger receptors on clodronate sensitive phagocytes. It seems

unlikely that 10F7 or 6A7 induce direct eryptosis of most cleared incompatible RBCs, as eryptotic RBCs would not re-enter circulation.

The current data differ somewhat from a previous characterization of clearance of hGPA RBCs by the 10F7 antibody.[26] Both the current and previous reports showed some effect of FcγR function on clearance; however, in the previous report FcγR played a significantly larger role in clearance. More importantly, the previous report indicated that clearance of hGPA RBCs by 10F7 was decreased in C3 KO mice. It is also worth noting that in the previous paper, 20% clearance still occurred in (C3 x FcγR) double KO mice. Thus, in agreement with the current findings, the previous study showed that some clearance was independent of either C3 or FcγRs; however, in the current studies, the vast majority of clearance was independent of either C3 or FcγRs. Although it is unclear why clearance was decreased in C3 KO mice in the previous study, but not in the current one, several factors may be relevant. First, the previous report modeled intraoperative transfusion in mice that were anesthetized and transfused through a surgically-exposed jugular vein; in contrast, the current study used tail vein infusion in unanesthetized mice. Inflammation can modify levels of circulating complement, FcγR function, and phagocyte function, in addition inflammation can increase antibody mediated RBC clearance.[50] Thus, surgery induced inflammation may have altered the biology of clearance in the previous report.[26] Second, RBC clearance was measured using 51-Cr labeling and sampling at a single post-transfusion time point; the current report uses two color fluorescent labeling and measurements by flow cytometry over 48 hours. Although the 51-Cr labeling method is analogous to the approach used in human studies, there is no control RBC population (e.g. such as the DiO labeled RBCs in this report) to control for differences in infusion volume or bleeding. Moreover, there were methodological differences in antibody purification, evaluation of purity, and

administration. Thus, the differences seen in the current system and the previous report may be due to methodological variation. Nonetheless, the current report demonstrates that conditions exist under which anti-hGPA mediated HTRs can proceed independent of both C3 and FcγR function.

Overall, our data are consistent with a model that entails a 2-phased clearing process. During the first phase, incompatible hGPA RBCs are rapidly sequestered outside the circulation after transfusion of incompatible RBCs. During the second phase, which occurs after 2 hours but before 24 hours, the "cleared" incompatible RBCs are ingested, thus making their clearance permanent (see Figure 2.7 for diagram). During the first phase of clearance, there is also a burst of serum cytokines. Although FcγRs are not required for RBC clearance, they are required for cytokine release, indicating that RBC bound IgG antibodies are ligating surface FcγRs and signaling into the cytokine secreting cells. Thus, when a sequestered IgG coated RBC encounters a phagocyte, two separate signaling events occur: 1) FcγRs are ligated resulting in cytokine burst, and 2) another signal (not through FcγR or C3R) induces phagocytosis.

Several therapeutics, targeting complement or FcγRs, have been proposed to inhibit RBC clearance. Although complement fixation on incompatible RBCs is well established in human transfusion biology,[51] and although FcγRs polymorphisms alter the efficiency of clearance of RBCs opsonized with anti-D in humans,[52] the causal role of complement and FcγRs has not been rigorously tested in the human setting of incompatible transfusions, and for various blood group antigens. Accordingly, to the extent that HTRs occur in humans independent of C3 or FcγRs, such therapies would likely fail to prevent removal of incompatible RBCs. Importantly, failure to prevent RBC clearance may not indicate complete lack of efficacy. For example, in the current system, although an FcγR blocking agent would not prevent RBC clearance, it could decrease morbidity/mortality

due to cytokine storm. Such an approach may have significant benefit in the context of autoimmune hemolytic anemia, even though it may not affect hematocrit. Thus, prevention of RBC clearance alone may be an overly simplified endpoint in drug trials in these settings. Accordingly, although C3 and FcγRs likely play important roles in many human HTRs, studies of alternate pathways are required to define the biology of HTRs completely; this will allow development of appropriate therapeutic interventions for HTRs, which may all have similar clinical presentations, but have distinct cellular and molecular mechanisms.

Acknowledgments

We would like to thank Nicole Smith for her excellent technical assistance. We also thank Kathryn Girard and Krystal Hudson for helping to blind micrsoscopy specimens and Chris Gilson for assisting in splenectomy of mice.

References:

1. Zimring JC, Spitalnik SL. Alloimmunization to Red Cell Antigens and Management of Alloimmunized Patients. In: Mintz PD, ed. Transfusion Therapy Clinical Principles and Practice. Bethesda Maryland: AABB Press, 2011:631-42.
2. Davenport RD. Management of Transfusion Reactions. In: Mintz PD, ed. Transfusion Therapy Clinical Principles and Practice. Bethesda Maryland: AABB Press, 2011:757-84.
3. Vamvakas EC, Blajchman MA. Transfusion-related mortality: the ongoing risks of allogeneic blood transfusion and the available strategies for their prevention. Blood 2009;**113**: 3406-17.
4. Linden JV, Paul B, Dressler KP. A report of 104 transfusion errors in New York State. Transfusion 1992;**32**: 601-6.
5. McClelland DB, Phillips P. Errors in blood transfusion in Britain: survey of hospital haematology departments. BMJ 1994;**308**: 1205-6.
6. Bowman J. The management of hemolytic disease in the fetus and newborn. Seminars in Perinatology 1997;**21**: 39-44.
7. Gehrs BC, Friedberg RC. Autoimmune hemolytic anemia. Am J Hematol 2002;**69**: 258-71.
8. Engelfriet CP. The immune destruction of red cells. Transfus Med 1992;**2**: 1-6.
9. Schreiber AD, Frank MM. Role of antibody and complement in the immune clearance and destruction of erythrocytes. I. In vivo effects of IgG and IgM complement-fixing sites. J Clin Invest 1972;**51**: 575-82.
10. Schreiber AD, Frank MM. Role of antibody and complement in the immune clearance and destruction of erythrocytes. II. Molecular nature of IgG and IgM complement-fixing sites and effects of their interaction with serum. J Clin Invest 1972;**51**: 583-9.

11. Petz LD, Garratty G. Immune Hemolytic Anemias. 2nd ed. Philadelphia: Churchill Livingstone, 2004.

12. Attanasio P, Shumilina E, Hermle T, Kiedaisch V, Lang PA, Huber SM, Wieder T, Lang F. Stimulation of eryptosis by anti-A IgG antibodies. Cell Physiol Biochem 2007;**20**: 591-600.

13. Chadebech P, Habibi A, Nzouakou R, Bachir D, Meunier-Costes N, Bonin P, Rodet M, Chami B, Galacteros F, Bierling P, Noizat-Pirenne F. Delayed hemolytic transfusion reaction in sickle cell disease patients: evidence of an emerging syndrome with suicidal red blood cell death. Transfusion 2009;**49**: 1785-92.

14. Brain MC, Pihl C, Robertson L, Brown CB. Evidence for a mechanosensitive calcium influx into red cells. Blood Cells Molecules and Diseases 2004;**32**: 349-52.

15. Brain M, Ruether B, Valentine K, Brown C, ter Keurs H. Life-threatening hemolytic anemia due to an autoanti-Pr cold agglutinin: evidence that glycophorin A antibodies may induce lipid bilayer exposure and cation permeability independent of agglutination. Transfusion 2010;**50**: 292-301.

16. Chadebech P, Michel M, Janvier D, Yamada K, Copie-Bergman C, Bodivit G, Bensussan A, Fournie JJ, Godeau B, Bierling P, Izui S, Noizat-Pirenne F. IgA-mediated human autoimmune hemolytic anemia as a result of hemagglutination in the spleen, but independent of complement activation and Fc alpha RI. Blood 2010;**116**: 4141-7.

17. Shibata T, Berney T, Reininger L, Chicheportiche Y, Ozaki S, Shirai T, Izui S. Monoclonal anti-erythrocyte autoantibodies derived from NZB mice cause autoimmune hemolytic anemia by two distinct pathogenic mechanisms. Int Immunol 1990;**2**: 1133-41.

18. Bigbee WL, Langlois RG, Vanderlaan M, Jensen RH. Binding specificities of eight monoclonal antibodies to human glycophorin A--studies with McM, and MkEn(UK)

variant human erythrocytes and M- and MNV-type chimpanzee erythrocytes. J Immunol 1984;**133**: 3149-55.

19. Bigbee WL, Vanderlaan M, Fong SS, Jensen RH. Monoclonal antibodies specific for the M- and N-forms of human glycophorin A. Mol Immunol 1983;**20**: 1353-62.

20. Blackall DP, Ugorski M, Pahlsson P, Shakin-Eshleman SH, Spitalnik SL. A molecular biologic approach to study the fine specificity of antibodies directed to the MN human blood group antigens. J Immunol 1994;**152**: 2241-7.

21. Taketo M, Schroeder AC, Mobraaten LE, Gunning KB, Hanten G, Fox RR, Roderick TH, Stewart CL, Lilly F, Hansen CT, et al. FVB/N: an inbred mouse strain preferable for transgenic analyses. Proc Natl Acad Sci U S A 1991;**88**: 2065-9.

22. Auffray I, Marfatia S, de Jong K, Lee G, Huang CH, Paszty C, Tanner MJ, Mohandas N, Chasis JA. Glycophorin A dimerization and band 3 interaction during erythroid membrane biogenesis: in vivo studies in human glycophorin A transgenic mice. Blood 2001;**97**: 2872-8.

23. Desmarets M, Cadwell CM, Peterson KR, Neades R, Zimring JC. Minor histocompatibility antigens on transfused leukoreduced units of red blood cells induce bone marrow transplant rejection in a mouse model. Blood 2009;**114**: 2315-22.

24. Wasniowska K, Lisowska E, Halverson GR, Chaudhuri A, Reid ME. The Fya, Fy6 and Fy3 epitopes of the Duffy blood group system recognized by new monoclonal antibodies: identification of a linear Fy3 epitope. Br J Haematol 2004;**124**: 118-22.

25. Unkeless JC. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J Exp Med 1979;**150**: 580-96.

26. Schirmer DA, Song SC, Baliff JP, Harbers SO, Clynes RA, Krop-Watorek A, Halverson GR, Czerwinski M, Spitalnik SL. Mouse models of IgG- and IgM-mediated hemolysis. Blood 2007;**109**: 3099-107.

27. Gilson CR, Zimring JC. Alloimmunization to transfused platelets requires priming of CD4+ T cells in the splenic microenvironment in a murine model. Transfusion 2011.

28. Hendrickson JE, Hod EA, Cadwell CM, Eisenbarth SC, Spiegel DA, Tormey CA, Spitalnik SL, Zimring JC. Rapid clearance of transfused murine red blood cells is associated with recipient cytokine storm and enhanced alloimmunogenicity. Transfusion 2011;**51**: 2445-54.

29. Zimring JC, Hair GA, Chadwick TE, Deshpande SS, Anderson KM, Hillyer CD, Roback JD. Nonhemolytic antibody-induced loss of erythrocyte surface antigen. Blood 2005;**106**: 1105-12.

30. Gros P, Milder FJ, Janssen BJ. Complement driven by conformational changes. Nat Rev Immunol 2008;**8**: 48-58.

31. Linton S. Animal models of inherited complement deficiency. Mol Biotechnol 2001;**18**: 135-48.

32. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell 1994;**76**: 519-29.

33. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity 2005;**23**: 41-51.

34. Kurlander RJ, Ellison DM, Hall J. The blockade of Fc receptor-mediated clearance of immune complexes in vivo by a monoclonal antibody (2.4G2) directed against Fc receptors on murine leukocytes. J Immunol 1984;**133**: 855-62.

35. Bonnerot C, Daeron M. Biological activities of murine low-affinity Fc receptors for IgG. Immunomethods 1994;**4**: 41-7.

36. Murphy JE, Tedbury PR, Homer-Vanniasinkam S, Walker JH, Ponnambalam S. Biochemistry and cell biology of mammalian scavenger receptors. Atherosclerosis 2005;**182**: 1-15.

37. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 1994;**174**: 83-93.

38. van Rooijen N, van Kesteren-Hendrikx E. Clodronate liposomes: perspectives in research and therapeutics. J Liposome Res 2002;**12**: 81-94.

39. Davenport RD, Burdick M, Moore SA, Kunkel SL. Cytokine production in IgG-mediated red cell incompatibility. Transfusion 1993;**33**: 19-24.

40. Hod EA, Cadwell CM, Liepkalns JS, Zimring JC, Sokol SA, Schirmer DA, Jhang J, Spitalnik SL. Cytokine storm in a mouse model of IgG-mediated hemolytic transfusion reactions. Blood 2008;**112**: 891-4.

41. Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, Lambris JD, Warner RL, Flierl MA, Hoesel LM, Gebhard F, Younger JG, Drouin SM, Wetsel RA, Ward PA. Generation of C5a in the absence of C3: a new complement activation pathway. Nat Med 2006;**12**: 682-7.

42. Fearon DT. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte. J Exp Med 1980;**152**: 20-30.

43. Cooper NR. Immune adherence by the fourth component of complement. Science 1969;**165**: 396-8.

44. Fearon DT. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. Proc Natl Acad Sci U S A 1979;**76**: 5867-71.

45. Medof ME, Iida K, Mold C, Nussenzweig V. Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes. J Exp Med 1982;**156**: 1739-54.

46. Garratty G. A new mechanism for immune destruction of red blood cells? Transfusion 2010;**50**: 274-7.

47. NIH conference. Pathophysiology of immune hemolytic anemia. Ann Intern Med 1977;**87**: 210-22.

48. Jandl JH, Kaplan ME. The destruction of red cells by antibodies in man. III. Quantitative factors influencing the patterns of hemolysis in vivo. J Clin Invest 1960;**39**: 1145-56.

49. Jones NC, Mollison PL, Veall N. Removal of incompatible red cells by the spleen. Br J Haematol 1957;**3**: 125-33.

50. Meite M, Leonard S, Idrissi ME, Izui S, Masson PL, Coutelier JP. Exacerbation of autoantibody-mediated hemolytic anemia by viral infection. J Virol 2000;**74**: 6045-9.

51. Hillyer CD SL, Ness PM, Anderson KC, Roback JD, editor *Blood Banking and Transfusion Medicine*. Second ed. Philadelphia: Churchill Livingstone, 2007.

52. Miescher S, Spycher MO, Amstutz H, De Haas M, Kleijer M, Kalus UJ, Radtke H, Hubsch A, Andresen I, Martin RM, Bichler J. A single recombinant anti-RhD IgG prevents RhD immunization: association of RhD-positive red blood cell clearance rate with polymorphisms in the FcgammaRIIA and FcgammaIIIA genes. Blood 2004;**103**: 4028-35.

Figure Legends

Figure 2.1: Analysis of RBC clearance during crossmatch incompatible transfusions. (A) Schematic of the hGPA x HOD RBCs used for incompatible transfusion studies. Histograms represent staining with anti-hGPA (10F7 or 6A7) or anti-Fy^3 (MIMA29) monoclonal antibodies of DiI-labeled hGPA x HOD RBCs (black line) or DiO-labeled control wild type FVB RBCs (shaded gray) after circulation in PBS treated C57BL/6 mice. **(B)** Representative dot plots of RBC survival at the 2 hour time point in mice passively immunized with anti-hGPA or anti-Fy^3 or controls treated with PBS alone. **(C)** Wild type mice were passively immunized with either anti-hGPA 10F7 (triangle), 6A7 (circle), or anti-Fy^3 (square) monoclonal antibodies; control mice received PBS alone (diamond, dashed line). All recipients were then transfused with a mixture of DiI-labeled hGPA x HOD RBCs and DiO-labeled FVB wild type RBCs. Percent survival of hGPA x HOD RBCs in passively immunized was normalized to hGPA x HOD RBC survival in PBS treated animals. Graph includes combined data from 6 independent experiments, each with 3 mice per group.

Figure 2.2: RBC clearance in C3 and FcγR KO mice. RBC survival was compared in wild-type C57BL/6 mice vs. C3KO mice **(A,B,C)** or vs. FcγR KO mice **(D,E,F).** Prior to transfusion, mice received either a control PBS injection (triangle) or were passively immunized with the indicated antibodies (squares). In all cases, KO mice are indicated by open symbols and dashed line, whereas C57BL/6 mice are indicated with closed symbols and a solid line. All recipients were transfused with a mixture of DiI-labeled hGPA x HOD RBCs and DiO-labeled wild type FVB RBCs. The same plots for PBS alone are represented in all 3 panels of indicated mouse type to allow comparison. Samples were collected 2 hrs, 20-24 hrs, and 2 days post transfusion and analyzed via

flow cytometry. Graphs include combined data from 3 independent experiments, each with 3 mice per group.

Figure 2.3: RBC clearance in C57BL/6 and C3 KO mice treated with an FcγR blocking antibody. Mice received control PBS or an anti-FcγR blocking antibody (2.4G2) prior to passive immunization with anti-hGPA (10F7 or 6A7) or anti-Fy^3 (MIMA29) (squares) or infusion of PBS alone (triangles) and transfusion of hGPA x HOD and wild type FVB RBCs labeled in different colors (as above). **(A)** 2 hour post-transfusion survival of hGPA x HOD RBCs in wild-type C57BL/6 recipient with (black solid) or without (dashed gray) 2.4G2 anti-Fc γ receptor antibody and indicated subsequent treatments. **(B,C,D)** Kinetics of RBC survival were compared in C3 KO mice receiving 2.4G2 (open symbols, dashed lines) or PBS (closed symbols, solid lines) and the indicated subsequent treatments. hGPA x HOD RBC survival was calculated as a function of wild-type FVB RBC survival. The same plots for PBS alone are represented in all 3 panels to allow comparison. Samples were collected 2 hrs, 20-24 hrs, and 2 days post transfusion and analyzed via flow cytometry. Graphs include combined data from 3 independent experiments, each with 3 mice per group.

Figure 2.4: The role of the spleen and phagocytes on RBC clearance and reappearance. (A-C) C57BL/6 mice were treated with either clodronate-filled liposomes (open symbols, dashed lines) or plain liposomes (closed symbols, solid lines). Recipients were either treated with PBS alone (triangles) or passively immunized with the indicated antibody (squares) prior to a transfusion of a mixture of DiI-labeled hGPA RBCs or HOD RBCs and DiO-labeled wild type FVB RBCs. **(D-F)** C57BL/6 were either splenectomized (open symbols, dashed line) or underwent sham surgery (closed symbols, solid line). Recipients were either treated with PBS alone (triangles) or

passively immunized with the indicated antibody (squares) prior to a transfusion of a mixture of DiI-labeled hGPA x HOD RBCs and DiO-labeled wild type FVB RBCs. hGPA x HOD, hGPA and HOD RBC survival were calculated as a function of wild-type FVB RBC survival. The same plots for PBS alone are represented in all 3 panels to allow comparison. Data are presented separately for each antibody: **(A,D)** 10F7, **(B,E)** 6A7, and **(C,F)** anti-Fy^3. Samples were collected at 2 hrs, 20-24 hrs, and 2 days post transfusion and analyzed via flow cytometry. Graphs include combined data from 3 independent experiments, each with 3 mice per group. **(G)** Circulating RBCs in the indicated groups and time points were stained with fluorescently labeled anti-mouse Igs. **(H)** Circulating RBCs in the indicated groups and time points were stained with indicated antibodies followed by fluorescently labeled anti-mouse Igs.

Figure 2.5: Anti-hGPA induces agglutination of circulating incompatible RBCs *in vivo* . (A) Representative ungated dot plots are shown of samples collected from mice treated with either PBS alone, anti-Fy^3 or anti-hGPA (10F7 or 6A7) immediately after transfusion and analyzed by flow cytometry. Gates are drawn on larger populations of events only seen in the presence of 10F7 or 6A7 **(B, C)** Gated large events are back reflected on the total population, indicating that large events are predominantly composed of DiI+ RBCs. **(D)** Percent survival of hGPA x HOD RBCs, normalized to hGPA x HOD RBC survival in PBS treated animals. **(E)** Percent of large complexes during early time points (immediately, 1 minute, 10 minutes, and 2 hours) post transfusion in animals treated with the indicated antibodies based upon gates shown in **(A)**. **(F)** Peripheral blood smears were produced from samples collected immediately after transfusion and were analyzed by confocal microscopy visualizing DiI-labeled hGPA x HOD RBCs (red) and DiO-labeled wild type FVB RBCs (green). Representative confocal images and flow cytometric plots are shown. Fields were chosen by an

observer blinded to the identity of the specimens. The experiment was repeated 3 times with similar results. Graphs include combined data from 3 independent experiments, each with 3 mice per group.

Figure 2.6: HTR induced inflammatory cytokine secretion is abrogated in FcγR KO mice as compared to C57BL/6 mice. Mice were passively immunized with either 10F7 or 6A7 **(A)** or anti-Fy^3 **(B)** prior to a transfusion of a mixture of DiI-labeled hGPA RBCs and DiO-labeled control FVB RBCs. Clearance patterns were determined to confirm proper functioning of antibodies (data not shown). Additional control mice were transfused with RBCs treated with phenylhydrazine prior to transfusion. All recipients were exsanguinated 2 hours post transfusion, sera were collected. Cytokines were quantified via flow cytometry. Experiment was performed 3 times, each with 3 mice per group.

Figure 2.7: Schematic of proposed mechanism of biphasic hemolytic transfusion reaction involving neither C3 nor Fc domains of bound antibodies binding to FcγRs.

Figure 2.1

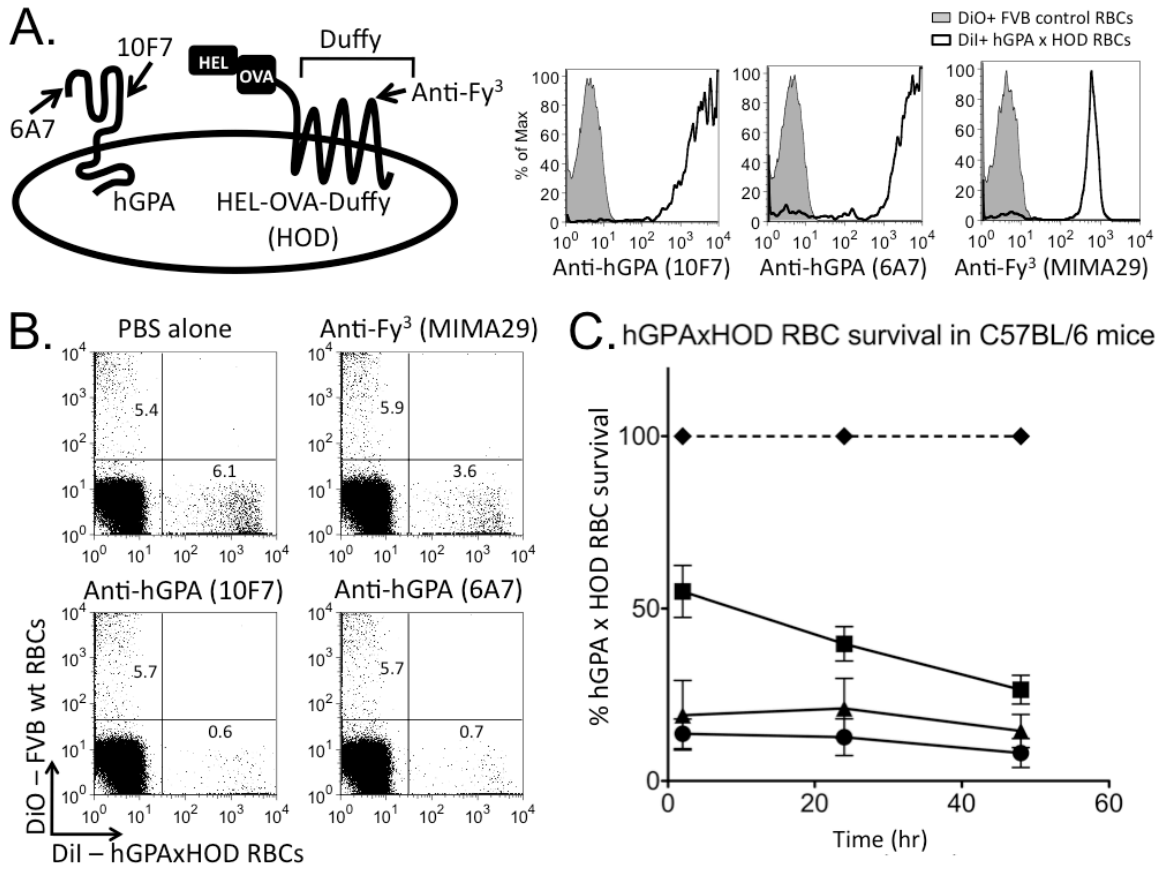

A.
10F7
6A7
HEL
OVA
Duffy
Anti-Fy3
hGPA
HEL-OVA-Duffy
(HOD)
DiO+ FVB control RBCs
DiI+ hGPA x HOD RBCs
% of Max
Anti-hGPA (10F7)
Anti-hGPA (6A7)
Anti-Fy3 (MIMA29)
B.
PBS alone
Anti-Fy3 (MIMA29)
5.4
6.1
5.9
3.6
Anti-hGPA (10F7)
Anti-hGPA (6A7)
5.7
0.6
5.7
0.7
DiO – FVB wt RBCs
DiI – hGPAxHOD RBCs
C. hGPAxHOD RBC survival in C57BL/6 mice
% hGPA x HOD RBC survival
Time (hr)

Figure 2.2

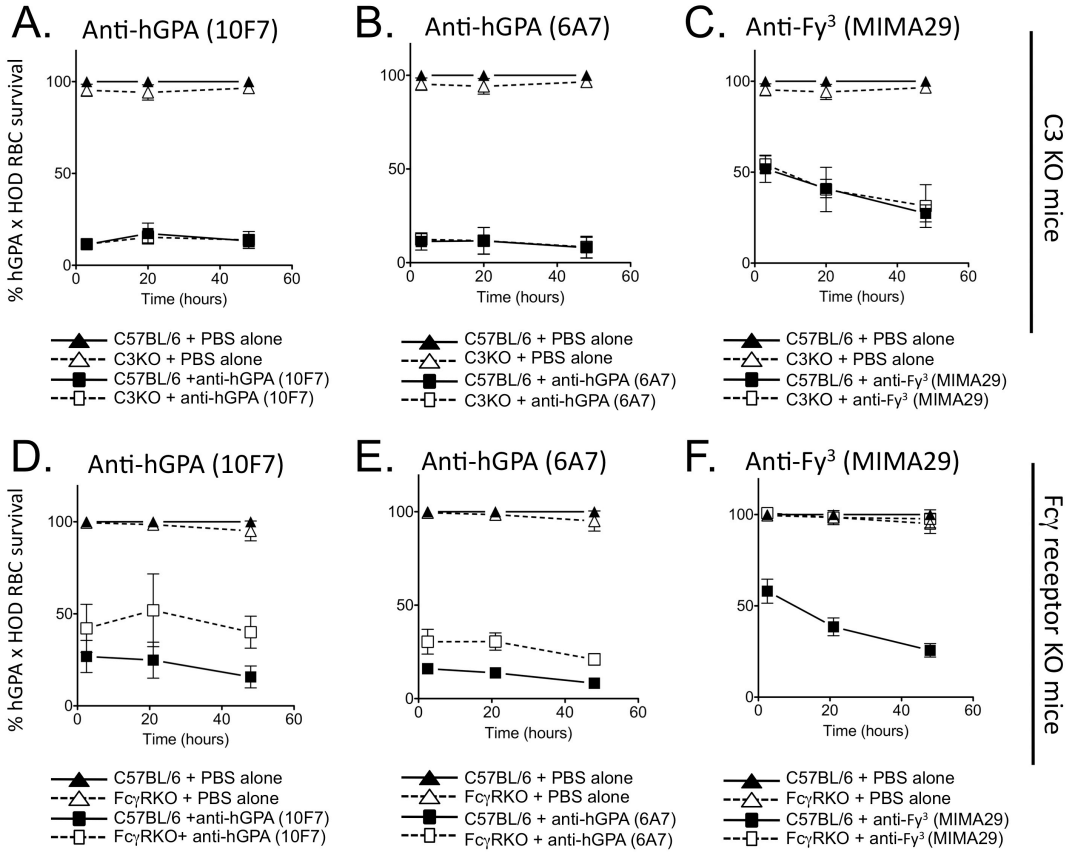

A. Anti-hGPA (10F7)
B. Anti-hGPA (6A7)
C. Anti-Fy³ (MIMA29)
% hGPA x HOD RBC survival
Time (hours)
C57BL/6 + PBS alone
C3KO + PBS alone
C57BL/6 +anti-hGPA (10F7)
C3KO + anti-hGPA (10F7)
C57BL/6 + anti-hGPA (6A7)
C3KO + anti-hGPA (6A7)
C57BL/6 + anti-Fy³ (MIMA29)
C3KO + anti-Fy³ (MIMA29)
C3 KO mice
D. Anti-hGPA (10F7)
E. Anti-hGPA (6A7)
F. Anti-Fy³ (MIMA29)
FcγRKO + PBS alone
FcγRKO+ anti-hGPA (10F7)
FcγRKO + anti-hGPA (6A7)
FcγRKO + anti-Fy³ (MIMA29)
Fcγ receptor KO mice

Figure 2.3

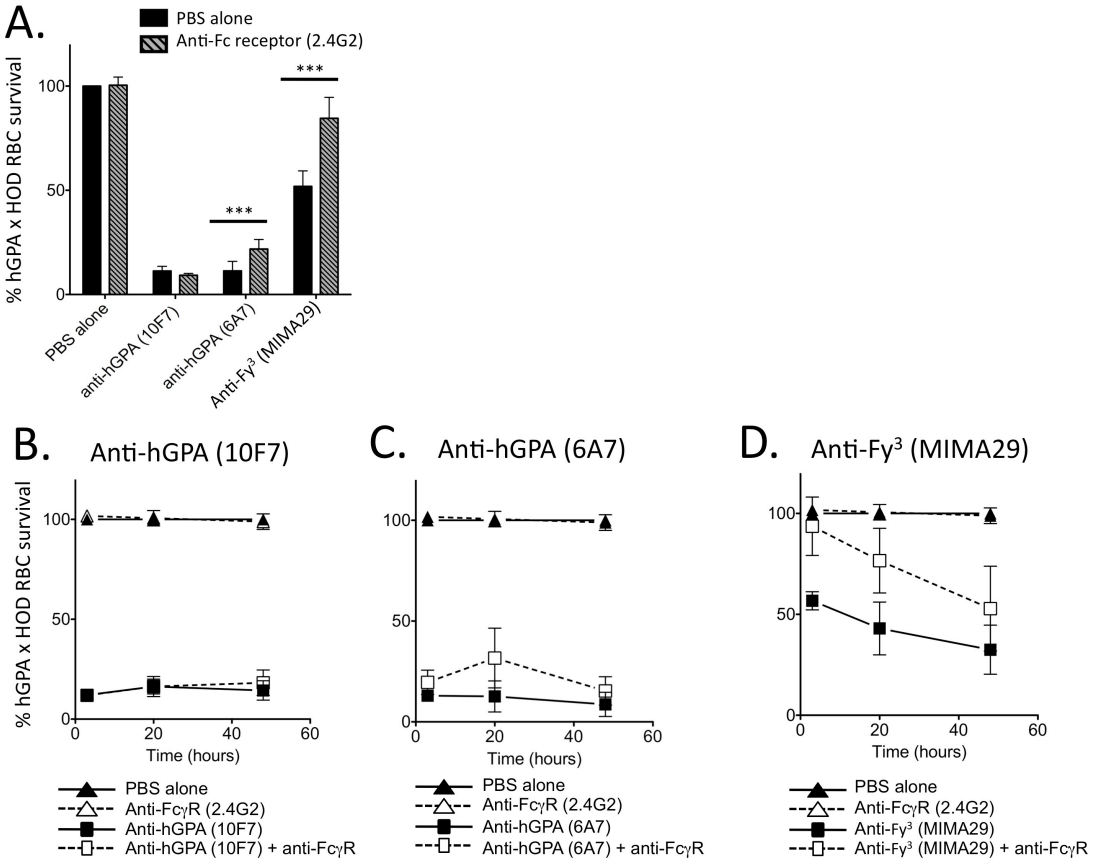

A.
PBS alone
Anti-Fc receptor (2.4G2)
% hGPA x HOD RBC survival
***
***
PBS alone
anti-hGPA (10F7)
anti-hGPA (6A7)
Anti-Fy3 (MIMA29)
B. Anti-hGPA (10F7)
C. Anti-hGPA (6A7)
D. Anti-Fy3 (MIMA29)
Time (hours)
PBS alone
Anti-FcγR (2.4G2)
Anti-hGPA (10F7)
Anti-hGPA (10F7) + anti-FcγR
Anti-hGPA (6A7)
Anti-hGPA (6A7) + anti-FcγR
Anti-Fy3 (MIMA29)
Anti-Fy3 (MIMA29) + anti-FcγR

Figure 2.4

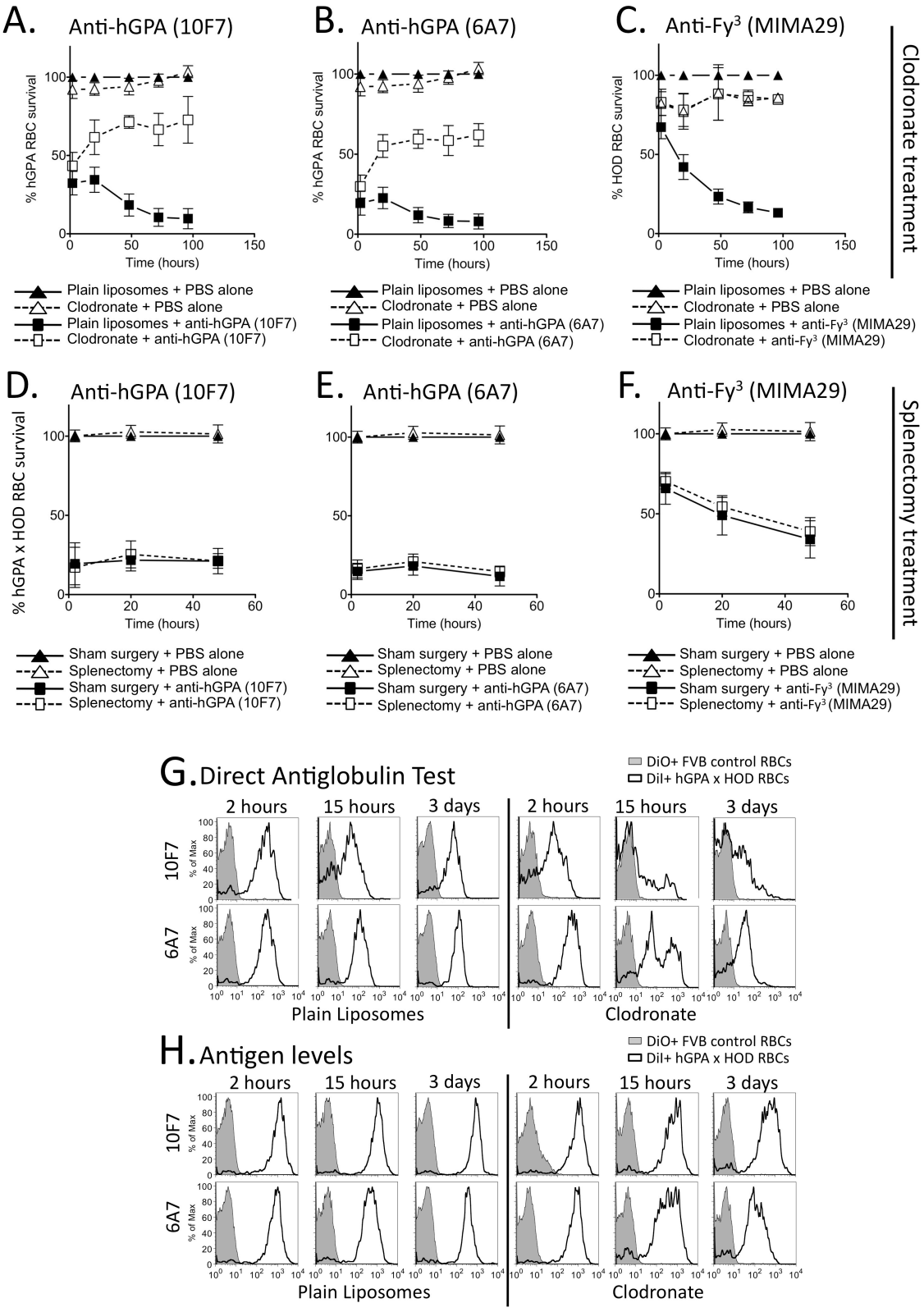

A. Anti-hGPA (10F7)
B. Anti-hGPA (6A7)
C. Anti-Fy3 (MIMA29)
Clodronate treatment
% hGPA RBC survival
% HOD RBC survival
Time (hours)
Plain liposomes + PBS alone
Clodronate + PBS alone
Plain liposomes + anti-hGPA (10F7)
Clodronate + anti-hGPA (10F7)
Plain liposomes + anti-hGPA (6A7)
Clodronate + anti-hGPA (6A7)
Plain liposomes + anti-Fy3 (MIMA29)
Clodronate + anti-Fy3 (MIMA29)
D. Anti-hGPA (10F7)
E. Anti-hGPA (6A7)
F. Anti-Fy3 (MIMA29)
Splenectomy treatment
% hGPA x HOD RBC survival
Sham surgery + PBS alone
Splenectomy + PBS alone
Sham surgery + anti-hGPA (10F7)
Splenectomy + anti-hGPA (10F7)
Sham surgery + anti-hGPA (6A7)
Splenectomy + anti-hGPA (6A7)
Sham surgery + anti-Fy3 (MIMA29)
Splenectomy + anti-Fy3 (MIMA29)
G. Direct Antiglobulin Test
DiO+ FVB control RBCs
DiI+ hGPA x HOD RBCs
2 hours
15 hours
3 days
10F7
6A7
% of Max
Plain Liposomes
Clodronate
H. Antigen levels

Figure 2.5

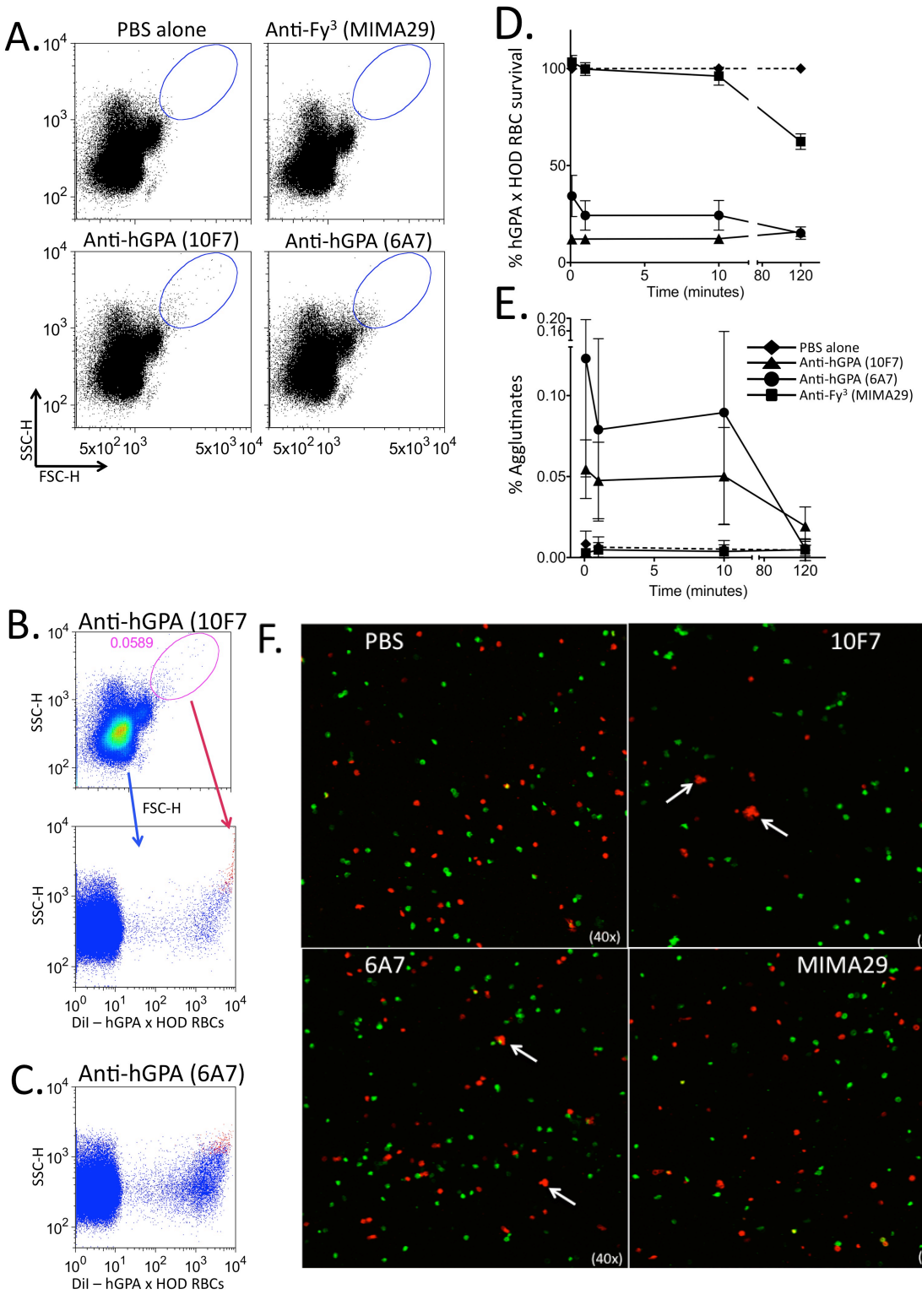

A.
PBS alone
Anti-Fy³ (MIMA29)
Anti-hGPA (10F7)
Anti-hGPA (6A7)
SSC-H
FSC-H
B.
Anti-hGPA (10F7
0.0589
DiI – hGPA x HOD RBCs
C.
Anti-hGPA (6A7)
DiI – hGPA x HOD RBCs
D.
% hGPA x HOD RBC survival
Time (minutes)
E.
% Agglutinates
PBS alone
Anti-hGPA (10F7)
Anti-hGPA (6A7)
Anti-Fy³ (MIMA29)
Time (minutes)
F.
PBS
10F7
6A7
MIMA29
(40x)

Figure 2.6

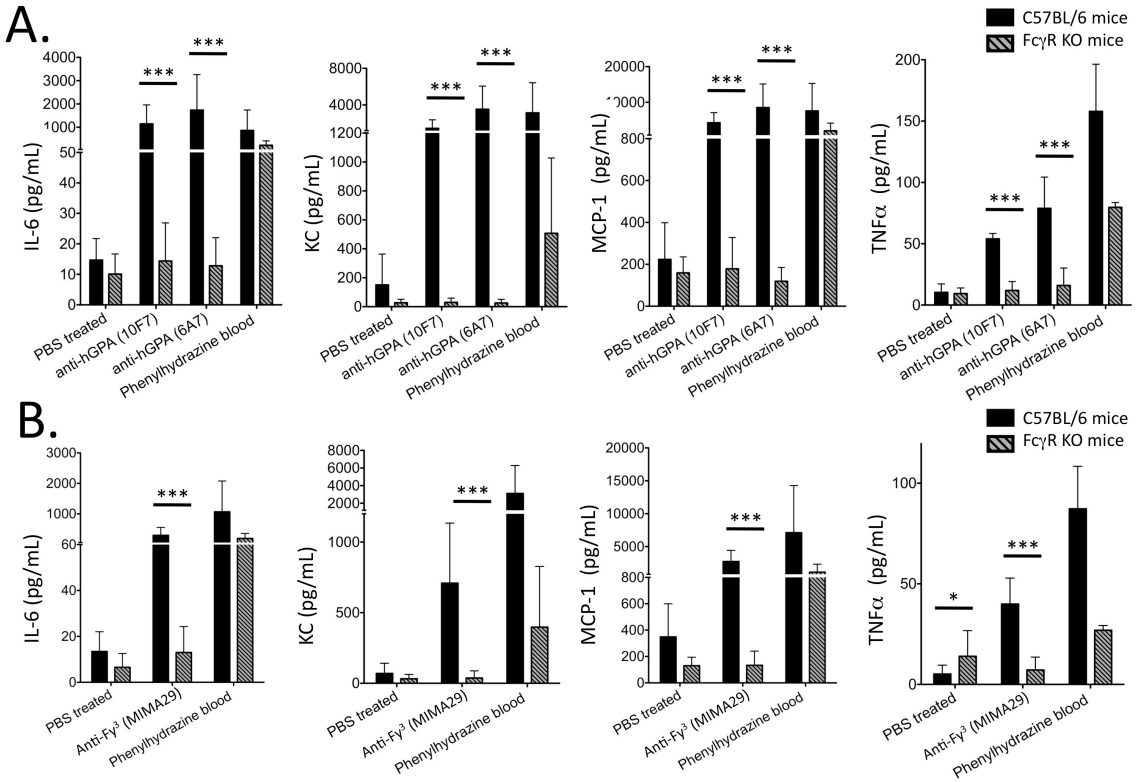
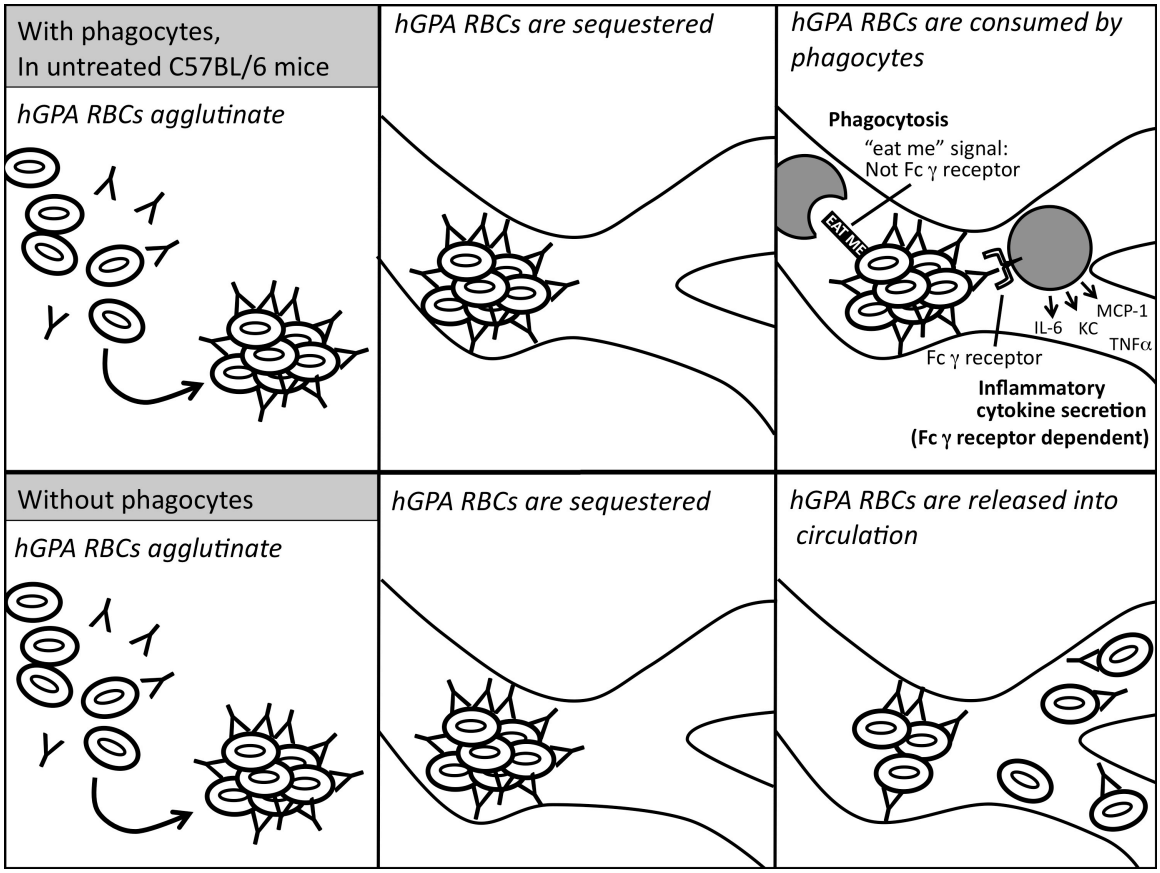

A.
C57BL/6 mice
FcγR KO mice
IL-6 (pg/mL)
KC (pg/mL)
MCP-1 (pg/mL)
TNFα (pg/mL)
***
PBS treated
anti-hGPA (10F7)
anti-hGPA (6A7)
Phenylhydrazine blood
B.
C57BL/6 mice
FcγR KO mice
IL-6 (pg/mL)
KC (pg/mL)
MCP-1 (pg/mL)
TNFα (pg/mL)
*
***
PBS treated
Anti-Fy3 (MIMA29)
Phenylhydrazine blood

Figure 2.7


With phagocytes,
In untreated C57BL/6 mice
hGPA RBCs agglutinate
hGPA RBCs are sequestered
hGPA RBCs are consumed by phagocytes
Phagocytosis
"eat me" signal:
Not Fc γ receptor
EAT ME
MCP-1
IL-6
KC
TNFα
Fc γ receptor
Inflammatory cytokine secretion
(Fc γ receptor dependent)
Without phagocytes
hGPA RBCs agglutinate
hGPA RBCs are sequestered
hGPA RBCs are released into circulation

Chapter 3

Resistance of a Subset of RBCs to Clearance by Antibodies in a Mouse Model of Incompatible Transfusion

Resistance of a Subset of RBCs to Clearance by Antibodies in a Mouse Model of Incompatible Transfusion

Justine S. Liepkalns[1], Chantel M. Cadwell[1], Sean R. Stowell[1], Eldad A. Hod[2], Steven L. Spitalnik[2], James C. Zimring[3*]

1. Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia 30322.
2. Department of Pathology and Cell Biology, Columbia University Medical Center, New York, NY, 10032 and Clinical Laboratories, New York-Presbyterian Hospital, New York, New York 10032
3. Puget Sound Blood Center Research Institute, Seattle, WA 98102

[*] To whom all correspondence should be addressed

Please address correspondence to:

James C. Zimring, M.D., Ph.D., Puget Sound Blood Center Research Institute, 1551 Eastlake Ave E, Seattle, WA 98102 jzimring@psbc.org

Word Count=3035

Abstract=200

Figure/table count= 4/0

Running Title: Hemolysis Resistance in Incompatible Transfusion

Abstract:

Alloimmunization to antigens on transfused RBCs represents the major barrier to chronic transfusion. In extreme cases of multiple alloimmunization, clinicians are faced with the decision of transfusing incompatible RBCs or risking death from lack of transfusion. The disastrous results of hemolytic transfusion reactions are well understood, and major pathways of clearance have been described. However, well described but poorly understood is the survival of a subset of incompatible donor RBCs during hemolysis, despite antibody binding. We utilize a tractable murine model of incompatible transfusion in which RBCs from transgenic donor mice expressing human glycophorin A are transfused into recipients passively immunized with anti-human glycophorin A. As in humans, the majority of RBCs are cleared; however, also like humans, a subset of incompatible donor RBCs persist in circulation, despite being bound by antibodies. Data contained here reject that lack of clearance is due to insufficient antibody or overwhelming of phagocytic machinery; rather, we establish that surviving RBCs represent a population resistant to clearance. Further data reject that resistance is due to C3 conversion to non-opsonizing C3dg or antigen-loss. In aggregate, these studies demonstrate for the first time that surviving RBCs during incompatible transfusion represent a population that is resistant to clearance.

Introduction

Exposure to a foreign blood group antigen by transfusion of red blood cells (RBCs) can initiate an immune response leading to the production of alloantibodies.[1-3] Due to this immunization, any further transfusion of RBCs sharing these same alloantigens is contraindicated due to the risk of incurring a potentially fatal hemolytic transfusion reaction (HTR). Despite efforts to match blood types to avoid such reactions, incompatible transfusions still occur. Due to the several hundred different human RBC blood group antigens described to date,[4,5] finding RBCs to transfuse with a matching blood type can be challenging and, at times, impossible. This is particularly problematic for patients with a rare blood type and/or who require chronic transfusions, resulting in alloantibodies against multiple antigens. If no compatible blood is available and the harm from withholding transfusion is dire, patients may be transfused with 'least incompatible' RBC units, which are given slowly while they are closely monitored for a HTR.[6]

Although HTRs are a clinical emergency when they occur, they are not the inevitable outcome of an incompatible transfusion. Although HTRs can occur for many antigens, they do not necessarily occur for every antigen. For example, only ~50% of ABO incompatible transfusions induce clinically-apparent HTRs.[7] In addition, alloantibodies to antigens in some blood group systems (e.g. Rhesus, Kell, and Kidd), will often, but not always, induce severe HTRs, whereas others (e.g. in the Lewis, Scianna, Gerbich, Cromer, Knops, and Chido/Rogers systems), seldom cause HTRs.[4] Why incompatible transfusions induce HTRs in some individuals, but not others remains unclear.

An additional complexity for understanding the biology of HTRs is the observation that, for a given incompatible transfusion, not all of the incompatible RBCs undergo the same fate. For example, the Direct Antiglobulin Test (DAT), which detects

donor RBCs coated with recipient antibodies, can remain positive days to weeks after an incompatible transfusion, even though a large number of the RBCs were initially destroyed during an HTR at the time of transfusion. Thus, some incompatible RBCs are rapidly cleared, whereas others survive long-term despite being antibody coated. The focus of the current studies is to analyze the biology of incompatible RBCs that persist in the circulation.

To study the resistance to clearance of antibody-coated RBCs, we transfused transgenic murine RBCs expressing the human glycophorin A (hGPA) antigen into mice passively immunized with 10F7, a monoclonal anti-hGPA antibody. A subset of these hGPA RBCs continued to circulate despite coating by anti-hGPA; this was not due to overloading of the reticuloendothelial system, exhaustion of the 10F7 antibody, or antigen-loss. Taken together, these results indicate that a subset of incompatible RBCs have a phenotype that makes them resistant to antibody-mediated clearance.

Methods

Mice

C57BL/6, FVB, and C3 knockout mice (C57BL/6 background) were purchased from the Jackson Laboratories (Bar Harbor, ME). hGPA transgenic mice, on an FVB background, were a generous gift from Dr. Narla Mohandas of the New York Blood Center. [8] C3 knockout and hGPA transgenic mice were bred by the Emory Division of Animal Resources. All mice were used at 8 to 14 weeks of age. All procedures were performed according to approved IACUC protocols.

Monoclonal antibody and passive immunization

The 10F7 anti-hGPA IgG monoclonal antibody was purified by protein G chromatography (Bio X Cell, West Lebanon, NH).[9-11] Wild type C57BL/6 mice were each passively immunized by tail vein injection with 500 µg of 10F7 diluted in 400 µl of phosphate buffered saline (PBS), or PBS alone, 2-5 hours prior to transfusion.

Fluorescent labeling and transfusion of murine RBCs

RBC labeling, transfusion, and tracking with DiO (3,3'-dihexadecyloxacarbocyanine perchlorate) and CM-DiI (chloromethylbenzamido 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate) was performed as previously described.[12] For DiD labeling, freshly collected blood from hGPA transgenic mice was labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) dissolved in DMSO. Fifty microliters of DiD (1 mg/ml in DMSO) was added to 1 ml of packed RBCs suspended in 10 ml PBS, incubated for 30 min at 37^{o}C, and then washed 3 times with PBS prior to mixing with other populations.

RBC survival studies

Post first transfusion: Blood samples were analyzed by flow cytometry from which a ratio of DiI-labeled hGPA RBCs to DiO-labeled wild-type FVB RBCs within each animal was calculated. This controlled for variations from volume transfused or volume loss during sampling, as such effects would equally affect both DiI and DiO populations. DiI:DiO ratios from PBS treated mice were set as 100% to control for any other alterations in RBC survival distinct from antibody mediated effects. DiI:DiO ratios in antibody treated mice were then normalized to DiI:DiO ratios in PBS treated mice.

Post second transfusion (see Figure 3.1A for explanation of experimental design): Ratios were calculated for both DiI:DiO ("experienced" hGPA RBCs:wild-type control RBCs) or DiD:DiO ("fresh" hGPA RBCs:wild-type control RBCs). Thus, the relative survival of both hGPA test populations (experienced vs. fresh) was normalized to wild-type DiO RBCs to control for any variation due to transfusion volume or loss during blood sampling. DiI:DiO and DiD:DiO ratios from PBS treated mice were set as 100% to control for any other alterations in RBC survival distinct from antibody mediated effects. DiI:DiO and DiD:DiO ratios from 10F7 treated mice were then normalized to DiI:DiO and DiD:DiO ratios from PBS treated mice. This allowed comparison of relative survival of "experienced" and "fresh" hGPA RBCs in the same animal.

RBC characterization

DATs were performed using polyclonal goat anti-mouse immunoglobulin (Ig) conjugated to allophycocyanin (BD biosciences Pharmingen), as previously described.[12] Antigen stains were performed using 10F7 and goat anti-mouse Ig as the secondary antibody. RBCs were also stained with anti-TER119 monoclonal antibody (BD bioscience Pharmingen) directly conjugated to allophycocyanin; a rat IgG2b kappa

antibody conjugated to allophycocyanin (BD bioscience Pharmingen) was used as an isotype-matched control.

Statistical analysis

RBC survival graphs were created with Prism software. All error bars represent one standard deviation.

Results

A subset of incompatible RBCs is resistant to antibody-mediated clearance.

To study RBC biology during an incompatible transfusion, we made use of a well-characterized, tractable model of antibody-mediated RBC clearance. [13,14] C57BL/6 recipients were made "incompatible" by passive immunization with a monoclonal anti-hGPA antibody (clone 10F7) diluted in PBS; control mice received PBS only. Recipients were then transfused with a 1:1 mixture of hGPA transgenic RBCs (labeled with DiI) and wild-type RBCs (labeled with DiO). Peripheral blood was collected from recipients at defined time points post-transfusion (see Figure 3.1A for experimental design). Recovery of the hGPA RBCs was normalized to recovery of wild-type RBCs to control for differences in transfusion volume and blood loss during sampling. Control mice receiving PBS alone without antibody were defined as exhibiting 100% RBC recovery; as a result, hGPA RBC survival in incompatible recipients was calculated as a fraction of that seen in PBS treated mice. This approach for monitoring RBC survival during incompatible transfusions was previously validated and described in detail.[12]

As previously observed,[14] 80% of hGPA RBCs were cleared in incompatible recipients by 2 hours post-transfusion; however, of the 20% of the RBCs that still circulated at 2 hours, no significant additional clearance was observed for up to 2 days post-transfusion (Figure 3.1B). Based on these data, we hypothesized that the surviving RBCs were resistant to clearance. However, several alternate explanations were equally consistent with the data, including: 1) that the continued survival of incompatible hGPA RBCs was due to insufficient administration of antibody during the passive immunization, and 2) that continued hGPA RBC survival was due to overloading the phagocytic capacity of the reticuloendothelial macrophage system.

These hypotheses were tested, as follows. Two days after the initial incompatible transfusion, recipient mice were exsanguinated and their RBCs were mixed with freshly

isolated, hGPA RBCs labeled with a third fluorescent dye (i.e. DiD) (see Figure 3.1A for experimental design). DiI, DiO, and DiD each fluoresce at different wavelengths, allowing for simultaneous analysis of three RBC populations. The mixed RBCs were then transfused into new recipients that had either been immunized with 10F7 or infused with PBS alone. Survival of fresh hGPA RBCs (labeled with DiI) or "experienced" hGPA RBCs (labeled with DiD) was normalized to survival of wild-type RBCs (labeled with DiO). The fresh hGPA RBCs again exhibited approximately 80% clearance. In contrast, no additional clearance was observed with the "experienced" hGPA RBCs (Figure 3.1B). Because the fresh and "experienced" hGPA RBCs circulated in the same animal, and because the fresh hGPA RBCs underwent 80% clearance, these results exclude both hypotheses: (1) that experienced hGPA RBCs survive due to diminished phagocytic capacity of the recipient, and (2) that insufficient concentrations of 10F7 antibody are present. Taken together, these data suggest that the 10F7 antibody-coated hGPA RBCs that survive the initial clearance exhibit the property of resistance.

Resistance to clearance of antibody-coated hGPA RBCs is not due to complement

Upon activation of the classical complement pathway by antibody binding, a reactive thioester moiety on C3 becomes available for covalent attachment to surrounding proteins, including the inciting antibody.[15] Such C3 binding can prevent recognition of the Fc domain of the antibody, thereby theoretically preventing phagocytosis.[16] As C3b can be rapidly converted to its degradation products (i.e. C3dg) that no longer opsonize RBCs via complement receptors, it has been hypothesized that complement binding can prevent Fc receptor mediated opsonization of antibody coated cells by blocking IgG-Fc receptor interactions. To test the hypothesis that complement modified products are responsible for the resistance to clearance of 10F7-coated hGPA

RBCs, the experimental approach described above was used in recipient mice with a targeted deletion of the C3 gene (i.e. C3 KO mice).

In C3 KO mice passively immunized with 10F7, 20% of the incompatible hGPA RBCs continued to circulate up to 2 days post transfusion, just as was observed in wild-type C57BL/6 mice (Figure 3.2A). To test for resistance to clearance, as above, blood was collected from exsanguinated recipients of after the first transfusion and mixed with fresh DiD-labeled hGPA RBCs (see experimental design in Figure 3.1A). Again, 60-80% of the incompatible fresh hGPA RBCs were cleared (Figure 3.2B). In contrast, "experienced" hGPA RBCs were not additionally cleared after the second transfusion into passively immunized C57BL/6 animals (Figure 3.2B). The same experimental design was also used using C3 KO mice as recipients of both the first and second transfusions, and similar results were observed (data not shown). These data reject the hypothesis that C3 and/or its break down products are required to induce the resistance phenotype of incompatible hGPA RBCs during an incompatible transfusion.

Characterization of Antigen Density and Antibody Binding of Resistant RBCs

Clearance-resistant hGPA RBCs were characterized by analyzing the surviving RBCs after the clearance phase of the 10F7 incompatible transfusion. Specimens were stained with an RBC specific antibody (i.e. anti-TER119) and the anti-hGPA antibody (10F7) (see Figure 3.1C-D for gating strategy). DiI-positive cells also stained positive for the TER119 RBC marker, which was specific because no staining was observed with the isotype matched control (Figure 3.3A). DiO-positive cells also stained positive for TER119 (data not shown). These data reject the hypothesis that the resistant cells represent non-erythrocytes that absorbed the fluorescent dye during the labeling process.

Staining with anti-hGPA revealed a positive shift only with DiI-positive RBCs and not with DiO-positive RBCs (Figure 3.3B), and this was observed over the entire 2 day time course studied. A small decrease in intensity of anti-hGPA staining was observed over time (Figure 3.3B); however, a large amount of hGPA antigen still remained on the surface of the DiI-positive RBCs. These data reject the hypothesis that the resistant RBC population was due to transfer of fluorescent dye to wild-type RBCs that do not express the hGPA antigen.

The persistence of *in vivo* bound 10F7 on transfused RBCs was tested by staining blood with secondary antibody (goat anti-mouse Igs). Compared to wild-type RBCs (i.e. DiO-positive), hGPA RBCs (i.e. DiI-positive) were reactive with anti-Igs throughout the entire 2 day time course, demonstrating surface bound immunoglobulins were only on hGPA RBCs. No binding of 10F7 or anti-Igs was detected on wild-type RBCs (i.e. DiO-positive) at any time point (Figure 3.3C). Staining intensity with secondary antibody alone was not as positive as antigen staining intensity with both anti-hGPA and secondary antibodies, and this difference became more apparent over time (Figure 3.3C). This observation demonstrates that not all 10F7 sites are occupied *in vivo*, suggesting gradual detachment of 10F7 over time.

Similar studies were performed on samples collected second transfusion. The hGPA cells remained positive for TER119 and continued to express the hGPA antigen (Figure 3.4B). The hGPA RBCs were reactive with anti-Igs only when samples were collected from 10F7-treated mice, but not in samples collected from PBS treated mice, even if the RBCs had survived in 10F7 treated animals during the first transfusion (Figure 3.4C). These data indicate that 10F7 binding is in a dynamic on-off equilibrium and that sufficient concentrations of circulating 10F7 are required for the continued presence of surface-bound antibodies coating the hGPA RBCs. As observed in the first transfusion, there is a slight decline in detectable hGPA RBCs over time, but an

abundance of antigen remains detectable over the time course of the experiment. (Figure 3.4C).

In PBS-treated mice, antigen levels were lower on hGPA RBCs that had been previously transfused into 10F7 passively immunized mice as compared to hGPA RBCs that had been previously transfused into PBS mice. However, no additional decrease was noted between hGPA RBCs circulating in PBS and 10F7 treated animals during the second transfusion (Figure 3.4B). In contrast, no difference was observed between PBS and 10F7 antibody treated animals of the second transfusion, when transfused with RBCs from animals treated with 10F7 during the first transfusion (Figure 3.4B). These observations support the concept that there is a decline in antigen levels upon the first *in vivo* encounter of RBCs with 10F7; however, no further decrease in antigen is observed in mice upon the second encounter with the same concentration of 10F7.

Discussion

We demonstrate in this manuscript that, very much like human RBC transfusions, murine RBCs undergo different fates after incompatible transfusion. Mouse RBCs expressing hGPA can be cleared, or can continue to circulate, after transfusion into recipients with circulating anti-hGPA antibodies. Above, we exclude the explanation of there simply being too little antibody to clear the remaining RBCs, by performing second transfusions into recipients freshly passively immunized with monoclonal antibody. We likewise rule out the explanation that the reticuloendothelial system's phagocytic capacity is simply exceeded, by showing that during the second transfusion, fresh, but not "experienced," hGPA RBCs are cleared, by the same reticuloendothelial system in individual recipients. Together, these data indicate that there are distinct populations of incompatible RBCs, with differential susceptibility to clearance; whether this is an intrinsic property of the RBCs or is a phenotype acquired over the course of transfusion is unclear.

Studies by Möller et al. suggested that resistance of RBCs during incompatible transfusion was induced by circulating complement.[17,18] Along similar lines, other studies demonstrated long-term survival of incompatible RBCs, with an initial organ sequestration of an incompatible RBC population.[19-23] Even though organ sequestration is not readily observed in our current study, it cannot be ruled out as a coincident mechanism, occurring below detectable levels and/or outside of the collection times. One suggested mechanism for organ sequestration is complement receptor binding to C3b with subsequent complement degradation, thereby releasing the RBC from the complement receptor.[24] Finally, Beum et al. showed that C3 binding can sterically hinder the Fc region of antibodies, which could prevent Fc gamma receptor-mediated erythrophagocytosis.[16] For these reasons, we directly tested whether C3 is required in

our system; however, because we observed identical biology in both wild-type and C3KO mice, we reject the hypothesis that C3 is required for the resistance phenotype in this system. Nonetheless, we cannot exclude a role for C3 in a redundant pathway.

We recently reported that the mechanism of clearance of hGPA RBCs by 10F7 requires neither complement nor Fc gamma receptors, and likely involves aggregation/sequestration, followed by a consumption signal other than opsonization by C3 or IgG (e.g. phosphatidylserine).[14] Although it is unclear to what extent this mechanism functions during incompatible transfusions in systems other than that involving hGPA, this background provides a unique advantage to the current studies, because it allowed for the study of complement-mediated resistance while not impeding the clearance mechanism of the transfused incompatible RBCs. The lack of clearance observed in this study in C3 KO mice is, therefore, not due to the absence of a major clearance system, but rather because of the continued resistance of the incompatible hGPA RBCs.

Monitoring of hGPA density on the RBCs demonstrated a subtle but significant decrease in hGPA levels over time post-transfusion. The significance of this decrease is unclear. It is worth noting that non-hemolytic loss of antigen, as a result of incompatible transfusion or autoantibodies, has been observed for a variety of human blood group antigens (although not for hGPA to the best of our knowledge). This antigen-loss process has been hypothesized to play a role in RBCs avoiding hemolysis. Although we cannot unequivocally rule out that the slight decrease in hGPA density on RBCs that survive incompatible transfusion is contributory to their survival, a substantial level of hGPA antigen persists. Indeed, RBCs are robustly cleared by antibodies recognizing antigens expressed at much lower levels than hGPA in various other murine systems.[25] Thus, it is unlikely that antigen-loss is a predominant mechanism of resistance in the current studies.

Because we recently reported that agglutination/sequestration appears to be the main mechanism by which hGPA RBCs are cleared by 10F7, we hypothesize that the phenotype of the resistant RBCs inhibits agglutination/sequestration in some, as yet undefined, way.[14] Alternatively, the resistant RBCs may transiently be cleared from and then re-enter the circulation due to a mechanism that avoids consumption. Future studies should address these issues in the context of the hGPA system and also investigate if a similar resistance biology is seen in other RBC antigen-antibody systems. Finally, it is important to understand the biology underlying why some incompatible RBCs continue to circulate in humans post-transfusion (e.g. persistent DAT-positive RBCs). A detailed understanding of this process may allow one to predict which incompatibilities are least dangerous in a given patient, to isolate units that are less prone to undergo hemolysis, and/or provide a rational basis for selecting and/or engineering RBCs that are resistant to antibody-mediated clearance.

Authorship and Conflict of Interest Statement: JSL performed the studies. JSL, CMC, SRS, EAH, SLS, and JCZ, conceived of the studies, designed experiments, interpreted data, and contributed to the writing of the manuscript. No authors have a conflict of interest to declare.

References

1. Blumberg N, Peck K, Ross K, Avila E. Immune response to chronic red blood cell transfusion. Vox Sang 1983;**44**: 212-7.
2. Heddle NM, Soutar RL, O'Hoski PL, Singer J, McBride JA, Ali MA, Kelton JG. A prospective study to determine the frequency and clinical significance of alloimmunization post-transfusion. Br J Haematol 1995;**91**: 1000-5.
3. Hoeltge GA, Domen RE, Rybicki LA, Schaffer PA. Multiple red cell transfusions and alloimmunization. Experience with 6996 antibodies detected in a total of 159,262 patients from 1985 to 1993. Arch Pathol Lab Med 1995;**119**: 42-5.
4. Reid M, Lomas-Francis C. The Blood Group Antigen Facts Book. 2nd ed. Amsterdam: Elsevier Academic Press, 2004.
5. Daniels G. Human blood groups. 2nd ed. Oxford: Blackwell Science, 2002.
6. Zimring JC, Spitalnik SL. Alloimmunization to Red Cell Antigens and Management of Alloimmunized Patients. In: Mintz PD, ed. Transfusion Therapy Clinical Principles and Practice. Bethesda : AABB Press, 2011:631-42.
7. Janatpour KA, Kalmin ND, Jensen HM, Holland PV. Clinical outcomes of ABO-incompatible RBC transfusions. Am J Clin Pathol 2008;**129**: 276-81.
8. Auffray I, Marfatia S, de Jong K, Lee G, Huang CH, Paszty C, Tanner MJ, Mohandas N, Chasis JA. Glycophorin A dimerization and band 3 interaction during erythroid membrane biogenesis: in vivo studies in human glycophorin A transgenic mice. Blood 2001;**97**: 2872-8.
9. Bigbee WL, Langlois RG, Vanderlaan M, Jensen RH. Binding specificities of eight monoclonal antibodies to human glycophorin A--studies with McM, and

MkEn(UK) variant human erythrocytes and M- and MNV-type chimpanzee erythrocytes. J Immunol 1984;**133**: 3149-55.

10. Bigbee WL, Vanderlaan M, Fong SS, Jensen RH. Monoclonal antibodies specific for the M- and N-forms of human glycophorin A. Mol Immunol 1983;**20**: 1353-62.

11. Blackall DP, Ugorski M, Pahlsson P, Shakin-Eshleman SH, Spitalnik SL. A molecular biologic approach to study the fine specificity of antibodies directed to the MN human blood group antigens. J Immunol 1994;**152**: 2241-7.

12. Zimring JC, Hair GA, Chadwick TE, Deshpande SS, Anderson KM, Hillyer CD, Roback JD. Nonhemolytic antibody-induced loss of erythrocyte surface antigen. Blood 2005;**106**: 1105-12.

13. Schirmer DA, Song SC, Baliff JP, Harbers SO, Clynes RA, Krop-Watorek A, Halverson GR, Czerwinski M, Spitalnik SL. Mouse models of IgG- and IgM-mediated hemolysis. Blood 2007;**109**: 3099-107.

14. Liepkalns JS, Hod EA, Stowell SR, Cadwell CM, Spitalnik SL, Zimring JC. Biphasic Clearance of Incompatible RBCs Through a Novel Mechanism Requiring Neither Complement nor Fc Gamma Receptors in a Murine Model. Transfusion in press.

15. Gros P, Milder FJ, Janssen BJ. Complement driven by conformational changes. Nat Rev Immunol 2008;**8**: 48-58.

16. Beum PV, Kennedy AD, Li Y, Pawluczkowycz AW, Williams ME, Taylor RP. Complement activation and C3b deposition on rituximab-opsonized cells

substantially blocks binding of phycoerythrin-labeled anti-mouse IgG probes to rituximab. J Immunol Methods 2004;**294**: 37-42.

17. Moller G. Antibody-Induced Depression of the Immune Response: A Study of the Mechanism in Various Immunological Systems. Transplantation 1964;**2**: 405-15.
18. Moller G. Isoantibody-Induced Cellular Resistance to Immune Haemolysis in Vivo and in Vitro. Nature 1964;**202**: 357-9.
19. Freedman J, Semple JW. Complement in Transfusion Medicine. In: Garratty G, ed. Immunobiology of Transfusion Medicine. New York: Marcel Dekker, 1994.
20. Atkinson JP, Frank MM. Studies on the in vivo effects of antibody. Interaction of IgM antibody and complement in the immune clearance and destruction of erythrocytes in man. J Clin Invest 1974;**54**: 339-48.
21. Brown DL, Lachmann PJ, Dacie JV. The in vivo behaviour of complement-coated red cells: studies in C6-deficient, C3-depleted and normal rabbits. Clin Exp Immunol 1970;**7**: 401-21.
22. Jandl JH, Jones AR, Castle WB. The destruction of red cells by antibodies in man. I. Observations of the sequestration and lysis of red cells altered by immune mechanisms. J Clin Invest 1957;**36**: 1428-59.
23. Schreiber AD, Frank MM. Role of antibody and complement in the immune clearance and destruction of erythrocytes. II. Molecular nature of IgG and IgM complement-fixing sites and effects of their interaction with serum. J Clin Invest 1972;**51**: 583-9.
24. Klein HG, Anstee DJ. Blood Transfusion in Clinical Medicine: Blackwell Publishing, 2005.

25. Campbell-Lee SA, Liu J, Velliquette RW, Halverson GR, Shirey RS, Chaudhuri A, Reid ME, Ness PM, Baldwin WM. The production of red blood cell alloantibodies in mice transfused with blood from transgenic Fyb-expressing mice. Transfusion 2006;**46**: 1682-8.

Figure legends

Figure 3.1 – A subset of hGPA RBCs are resistant to antibody-mediated clearance after incompatible transfusion

(A) Schematic of experimental design for studies of transfusions of incompatible hGPA RBCs. **(B)** first transfusion: Wild-type mice were either passively immunized with affinity-purified 10F7 anti-hGPA monoclonal antibody diluted in PBS (squares, solid line) or received PBS alone (diamonds, dashed line). All recipients were then transfused with a mixture of DiI-labeled hGPA RBCs and DiO-labeled wild-type FVB RBCs. Second transfusion: recipients from the first transfusion were exsanguinated, their RBCs were mixed with freshly obtained DiD-labeled hGPA RBCs, and then the samples were transfused into C57BL/6 mice that either received PBS alone (diamonds, dashed line) or were passively immunized with 10F7 (squares, solid line). **(C)** Gating strategy used for the post-transfusion analysis of circulating RBCs with representative flow cytometric plots. **(D)** histograms represent staining with anti-hGPA on the indicated gated populations (DiI+ (hGPA) or DiO+ (wild-type)). Graphs include combined data from 3 independent experiments, each with 5-6 mice per group.

Figure 3.2 – Clearance resistance of hGPA incompatible RBCs in C3 KO mice

(A) First transfusion: C3 KO mice were passively immunized with anti-hGPA (squares, solid line) or infused with PBS alone (diamonds, dashed line). All recipients were then transfused with a mixture of DiI-labeled hGPA RBCs and DiO-labeled FVB wild-type RBCs. Percent survival of hGPA RBCs was normalized to hGPA RBC survival in PBS-treated animals. **(B)** Second transfusion: transfused recipients from the first transfusion were exsanguinated, the resulting RBCs were mixed with freshly isolated DiD-labeled hGPA RBCs, and then transfused into C3KO mice treated with either PBS (diamonds,

dashed line) or 10F7 (squares, solid line). Graphs include combined data from 3 independent experiments, each with 3 mice per group.

Figure 3.3 – Persistence of antigen and antibodies on hGPA incompatible RBCs

(A) Histogram represents staining with anti-Ter119 (black line) or isotype matched control (shaded gray) of post first transfusion after gating on DiI-hGPA (Experienced) RBCs. **(B)** Histograms represent staining with anti-hGPA (10F7) monoclonal antibodies followed by secondary anti-mouse IgGs after gating on DiI-labeled hGPA RBCs (black line) or DiO-labeled control wild type FVB RBCs (shaded gray). **(C)** Histograms represent staining with secondary anti-mouse IgGs alone (DAT) of DiI-labeled hGPA RBCs (black line) or DiO-labeled control wild type FVB RBCs (shaded gray). In all cases, recipient animals and times post-transfusion are indicated. This experiment has been performed 3 separate times with similar results; representative histograms are shown.

Figure 3.4 – Antigen and antibodies during second transfusion in C57BL/6 mice

(A) The histogram represents staining with anti-TER119 (black line) or and isotype-matched control (shaded gray) of cells obtained after the second transfusion after gating on DiI-positive hGPA (i.e. “experienced”) RBCs. **(B)** The histograms represent staining with the anti-hGPA 10F7 monoclonal antibody and secondary anti-mouse Ig after gating on DiI-labeled hGPA RBCs (black line) or DiO-labeled control wild-type FVB RBCs (shaded gray). **(C)** The histograms represent staining with secondary anti-mouse Ig alone (i.e. a DAT) after gating on DiI-labeled hGPA RBCs (black line) or DiO-labeled control wild-type FVB RBCs (shaded gray). In all cases, recipient mice and times post-transfusion are indicated. This experiment was performed 3 separate times with similar results; representative histograms are shown.

Figure 3.1

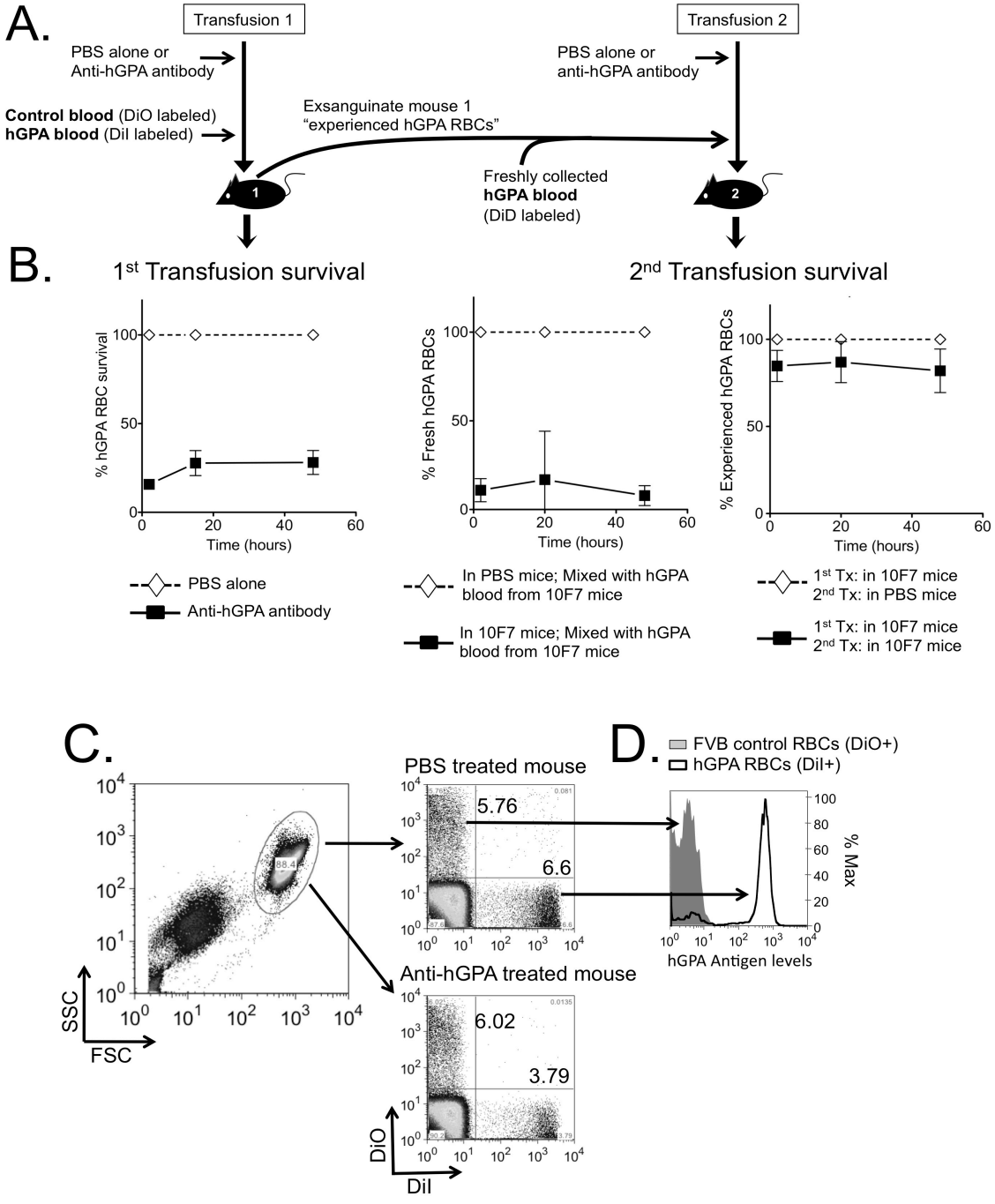

A.
Transfusion 1
Transfusion 2
PBS alone or
Anti-hGPA antibody
PBS alone or
anti-hGPA antibody
Control blood (DiO labeled)
hGPA blood (DiI labeled)
Exsanguinate mouse 1
"experienced hGPA RBCs"
Freshly collected
hGPA blood
(DiD labeled)
1
2
B.
1st Transfusion survival
2nd Transfusion survival
% hGPA RBC survival
% Fresh hGPA RBCs
% Experienced hGPA RBCs
Time (hours)
PBS alone
Anti-hGPA antibody
In PBS mice; Mixed with hGPA blood from 10F7 mice
In 10F7 mice; Mixed with hGPA blood from 10F7 mice
1st Tx: in 10F7 mice
2nd Tx: in PBS mice
1st Tx: in 10F7 mice
2nd Tx: in 10F7 mice
C.
SSC
FSC
PBS treated mouse
5.76
6.6
Anti-hGPA treated mouse
6.02
3.79
DiO
DiI
D.
FVB control RBCs (DiO+)
hGPA RBCs (DiI+)
% Max
hGPA Antigen levels

Figure 3.2

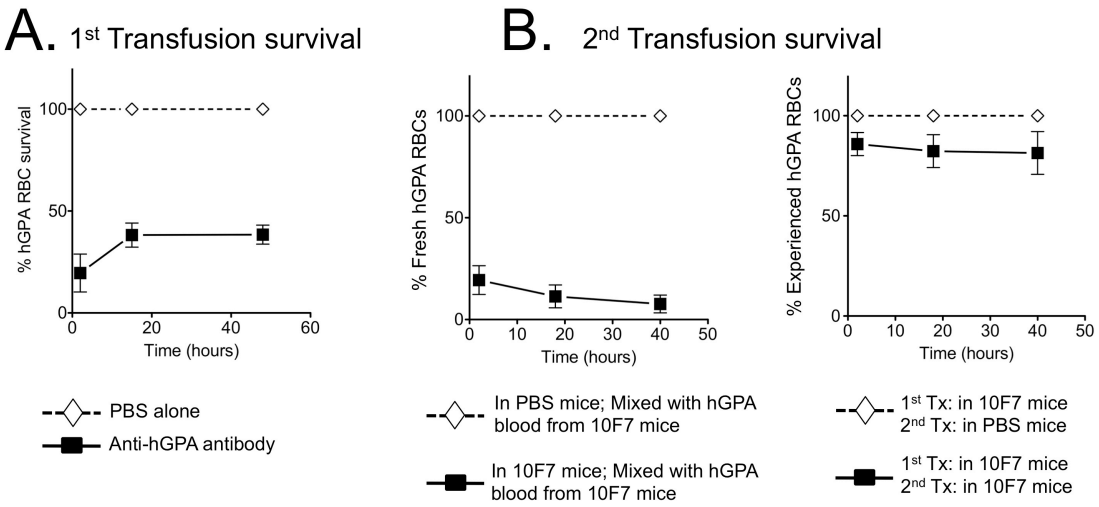

A. 1st Transfusion survival
B. 2nd Transfusion survival
% hGPA RBC survival
Time (hours)
PBS alone
Anti-hGPA antibody
% Fresh hGPA RBCs
Time (hours)
In PBS mice; Mixed with hGPA blood from 10F7 mice
In 10F7 mice; Mixed with hGPA blood from 10F7 mice
% Experienced hGPA RBCs
Time (hours)
1st Tx: in 10F7 mice
2nd Tx: in PBS mice
1st Tx: in 10F7 mice
2nd Tx: in 10F7 mice

Figure 3.3

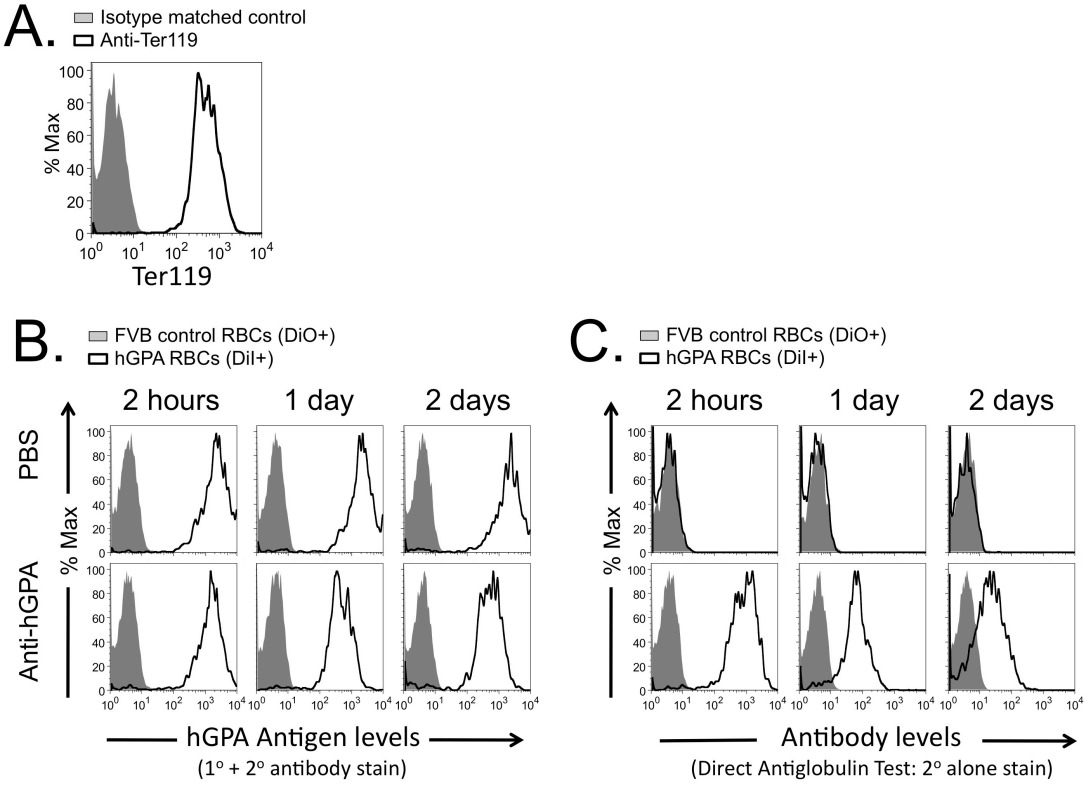
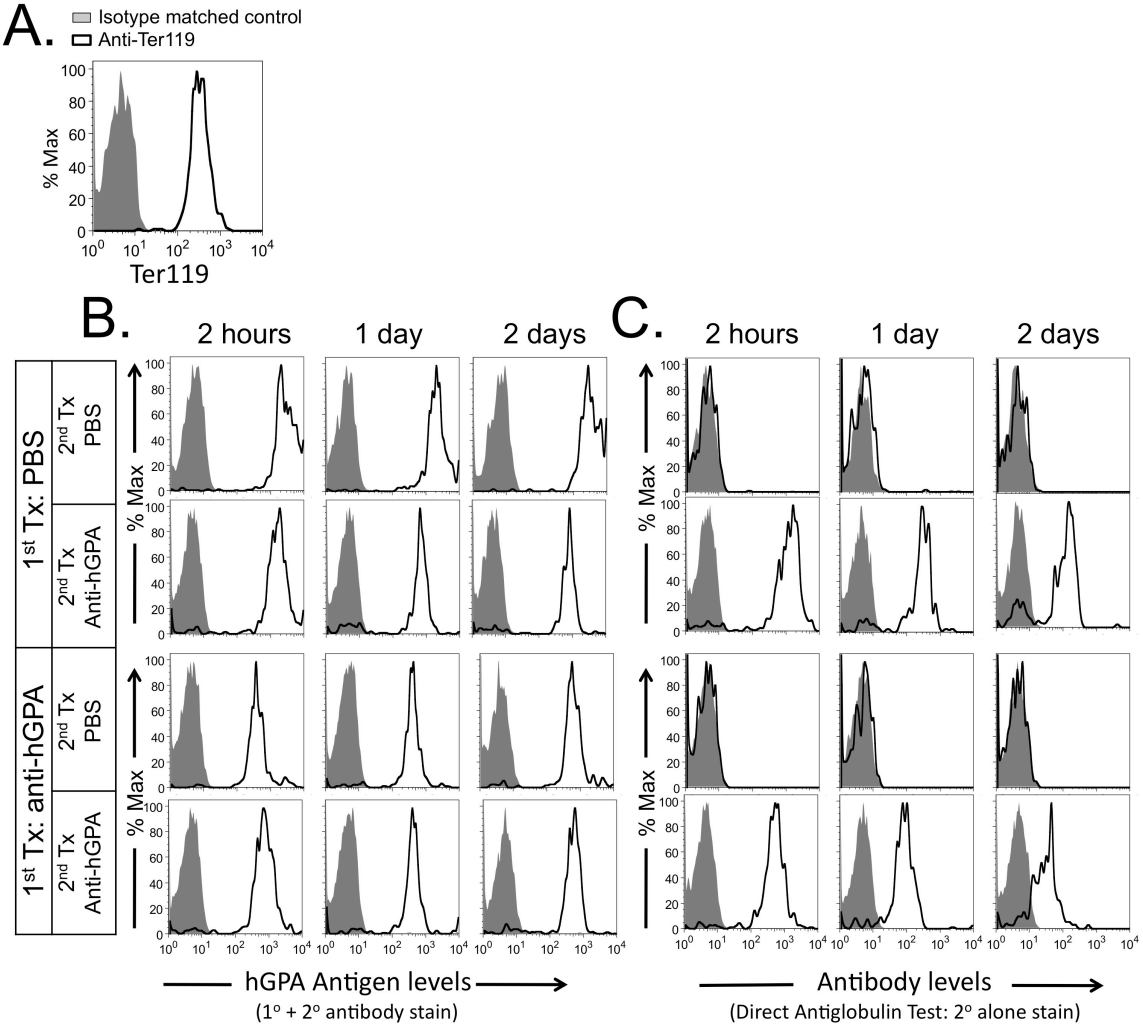

A.
Isotype matched control
Anti-Ter119
% Max
Ter119
B.
FVB control RBCs (DiO+)
hGPA RBCs (DiI+)
2 hours
1 day
2 days
PBS
Anti-hGPA
% Max
hGPA Antigen levels
(1° + 2° antibody stain)
C.
FVB control RBCs (DiO+)
hGPA RBCs (DiI+)
2 hours
1 day
2 days
% Max
Antibody levels
(Direct Antiglobulin Test: 2° alone stain)

Figure 3.4


A.
Isotype matched control
Anti-Ter119
% Max
Ter119
B.
C.
2 hours
1 day
2 days
1st Tx: PBS
1st Tx: anti-hGPA
2nd Tx PBS
2nd Tx Anti-hGPA
hGPA Antigen levels
(1° + 2° antibody stain)
Antibody levels
(Direct Antiglobulin Test: 2° alone stain)

Chapter 4

Resistance of Duffy expressing RBCs does not require complement C3 but is instead innate to the persistent population

Resistance of Duffy expressing RBCs does not require complement C3 but is instead innate to the persistent population

Justine S. Liepkalns[1], James C. Zimring[1,2*]

1. Center for Transfusion and Cellular Therapies, Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia 30322.

2. Aflac Cancer Center, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, 30322

[*] To whom all correspondence should be addressed

Please address correspondence to:

James C. Zimring, M.D., Ph.D., Emory University School of Medicine, Woodruff Memorial Building Suite 7107, 101 Woodruff Circle, Atlanta, GA 30322, USA (Telephone 404-712-2174, Fax 404-727-5764) Email jzimrin@emory.edu

Word Count= 3905

Abstract= 280

Figure/table count= 6/0

Running Title: Hemolysis Resistance in Incompatible Transfusion

Abstract

Incompatible red blood cells (RBCs) do not always clear and/or lead to potentially fatal outcomes, including hemolytic transfusion reactions (HTRs), hemolytic disease of the fetus and newborn (HDFN) and autoimmune hemolytic anemia (AIHA). Complement mediated RBC protection has been suggested as a mechanism of resistance during incompatible transfusions. In the current report, we analyzed mechanisms of RBC survival during incompatible transfusions of murine RBCs expressing a fusion protein Hen-egg-lysozyme, Ovalbumin, and human Duffy (HOD) into mice with anti-Fy^3 antibodies. C3 and Fcγ receptor knockout (KO) mice along with wild type mice were passively immunized with anti-Fy^3 antibodies (MIMA29) and transfused with a mixture of control RBCs and RBCs expressing the HOD antigen. Recipients were then exsanguinated and blood was mixed with fresh HOD RBCs and subsequently transfused into anti-Fy^3 antibody treated wild type mice. Post transfusion blood was collected and analyzed via flow cytometry. This model of incompatible transfusion into wild type or C3 KO antibody-treated mice both resulted in resistance of a population of HOD RBCs. Transfusion into Fcγ receptor KO mice resulted in no clearance during the 1^{st} transfusion and clearance in the 2^{nd} transfusion of the majority of the incompatible HOD RBCs. Resistant incompatible HOD RBCs in wild type, C3KO and Fcγ receptor KO mice were positive for RBC marker, Fy^3 antigen and surface-bound antibodies. Diminishing antigen expression and antibody binding was only noticed in wild type and C3 KO mice. These data demonstrate the existence of a resistant incompatible HOD RBC population. This resistance does not required C3, which contradicts the suggested complement mediated mechanism of resistance. Moreover, incompatible HOD RBC resistance was not found to be acquired but was instead intrinsic to the circulating RBCs.

Introduction

There have now been several hundred blood group antigens described on the human red blood cell (RBC).[1] In most cases, once a patient is immunized against one of these blood group antigens, a second exposure to the same antigen can lead to a severe hemolytic transfusion reaction (HTR). In the case of ABO however, antibodies against these blood group antigens arise within the first few months of life, therefore RBC units are always crossmatched for this blood group prior to transfusion. Despite the exhaustive efforts of matching blood units to patients, incompatible transfusions still occur due mostly to either mistransfusions[2] or lack of existing matching units, particularly in the case of patients in need of multiple transfusions[3] such as those with leukemia,[4] thalassemia,[5] Myelodysplastic syndrome[6] or sickle cell anemia.[7]

The intricacies of incompatible transfusions are still not understood. It has been reported that HTRs are not the inevitable outcome of an incompatible transfusion. Not all ABO incompatible transfusions lead to HTRs.[8] Partial survival of incompatible RBCs has also been observed in other blood group systems.[9-13] In addition, patients with autoimmune hemolytic anemia (AIHA) still have circulating RBCs, some found to have bound antibodies.[14,15]

In order to study incompatible RBC populations during transfusions we utilized a transgenic mouse expressing a RBC specific fusion protein containing Hen egg lysozyme, Ovalbumin and the human blood group Duffy (HOD). It has been shown that incompatible HOD RBCs clear via the Fcγ receptor pathway (See chapter 2 of thesis). In this current study, HOD expressing RBCs were transfused into passively immunized mice to simulate an incompatible transfusion in a murine model. A portion of the incompatible HOD RBCs was found to not clear and persist in circulation up to 2 days post transfusion. The survival of incompatible HOD RBCs was found to not be due to saturation of the reticuloendothelial system. Staining of the resistant HOD RBCs revealed a population of incompatible RBCs expressing varying levels of HOD on their surfaces. Furthermore, we demonstrated that resistance was not due to C3 and was instead due to the intrinsic nature of the HOD RBCs.

Methods

Mice

C57BL/6 (B6), FVB, and C3 knockout mice were purchased from the Jackson Laboratories (Bar Harbor, ME). FcγR KO mice (Fcer1g) were purchased from Taconic Farms, Inc. All mice were used at 8 to 14 weeks of age. HOD mice consisted of transgenic animals (FVB background) expressing a fusion protein Hen Egg Lysozyme (HEL), Ovalbumin (Ova), and Duffy b (aka. HOD mice)[16] (see figure 4.1A for schematic of antigen). All breeding (including FcγR KO and C3 KO mice) was performed by the Emory University Department of Animal Resources Husbandry Services and all procedures were performed according to approved IACUC protocols.

Antibodies and passive immunization

Anti-Fy^3 (aka: anti-HOD, MIMA29) a generous gift from Marion Reid and Greg Halverson at the New York Blood Center is an IgG2a. Antibodies were purified by protein G chromatography (Bio X Cell, West Lebanon, NH). The wild type C57BL/6 mice were passively immunized with 500μl of 200μg MIMA29 in PBS or were given PBS alone by tail vein injection 2-5 hours prior to transfusion.

Fluorescent labeling and transfusion of murine RBCs

HOD transgenic mice and wild type FVB mice were anesthetized with isoflurane and exsanguinated by enucleation. Blood collected was washed with phosphate buffered saline (PBS) and labeled with one of 3 different lipophilic dyes used to track the survival of these RBCs post transfusion. HOD blood was typically labeled with 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI), and FVB blood was typically labeled with 3,3'-dihexadecyloxacarbocyanine perchlorate (DiO). After labeling, HOD and wild type FVB blood cells were mixed at a 1:1 ratio and brought to a 20% hematocrit with LPS-free PBS. Each

C57BL/6 recipient was transfused by tail-vein injection, with 500μl of above prepared blood mixture.

Two days post transfusion the mice were exsanguinated by enucleation and blood was mixed with freshly collected HOD blood labeled with a different lipophilic dye, typically 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD). The fresh DiD-labeled HOD blood ("fresh HOD blood") was mixed with blood collected from recipients of the 1st transfusion at a 1:1 ratio with the surviving DiI-labeled HOD RBCs ("experienced HOD blood"). The total mixture was brought to a 20% hematocrit with LPS-free PBS and 500μl was transfused by tail vein injection into fresh C57BL/6 mice.

RBC survival

After both the first and second transfusions, mice were anesthetized and 25μl to 50μl of blood was collected retroorbitally at 2 hours, 20 hours, and 2 days post transfusion. After the first transfusion, samples were analyzed by flow cytometry from which a ratio of DiI-labeled HOD blood to DiO-labeled FVB blood within each animal was calculated. These ratios from antibody treated animals were then normalized to PBS treated animals and graphed as percent survival.

After the second transfusion, the ratio of DiI-labeled "experienced" HOD blood cells to DiO-labeled FVB blood cells was calculated and the resulting ratios were normalized to the ratios calculated from RBCs transfused into 2 consecutive PBS treated mice. Similarly, the ratio of DiD-labeled "fresh" HOD RBCs (never previously transfused) to DiO-labeled FVB RBCs was calculated. However, these DiD-HOD to DiO-FVB RBC ratios were each normalized to their PBS counterparts transfused with the same blood mixture (both originated from the same 1st transfusion recipients).

RBC characterization

A Direct Antiglobulin Test (DAT) was performed on samples collected post first and second transfusions at a 1:100 dilution of goat anti-mouse Igs antibodies (BD biosciences). The samples were also stained with a primary antibody, MIMA29, at a concentration of 1μg/100μl of Fluorescence-activated cell sorting (FACS) buffer then with a 2^{o} antibody, goat anti-mouse Igs (BD bioscience Pharmingen), diluted 1:100 in FACS buffer. The samples were also stained with Rat anti-TER119 (BD bioscience Pharmingen) also diluted to 1:100 with FACS buffer; Rat IgG2b k (BD bioscience Pharmingen) antibodies were used as an isotype matched control.

Calculations and Statistical analysis.

Survival graphs were all calculated and created with Prism software. All error bars represent one standard deviation.

Results

A subset of incompatible HOD RBCs is resistant to antibody-mediated clearance and continues to circulate during a subsequent transfusion

To study RBC biology during an incompatible transfusion, we made use of a well-characterized tractable model of antibody mediated RBC clearance (refer to dissertation chapters 2 and 3).[17] C57BL/6 recipients were passively immunized with monoclonal anti-Fy^3 antibody followed by transfusion with RBCs from transgenic mice expressing a RBC-specific HOD antigen (see figure 4.1A for schematic of antigen).[16] To allow tracking of RBC survival, prior to transfusion, a fluorescent labeling approach was utilized (also see dissertation chapters 2 and 3). HOD RBCs and wild-type FVB RBCs were labeled with fluorescent dyes, DiI and DiO, respectively. A mixture of the labeled HOD and wild-type RBCs was transfused and blood was collected at the indicated time-points post-transfusion (see figure 4.1B for experimental design). Survival of HOD RBCs was calculated as a function of wild-type FVB RBC survival, so as to control for mouse-to-mouse volume variations regarding transfusion and specimen bleeding. All survival of HOD RBCs in experimental animals was normalized to baseline survival established in PBS treated mice (see figure 4.1C for representative plots and gating strategy). About 40% of HOD RBCs were cleared rapidly in incompatible recipients within 2 hours post transfusion (Figure 4.1D). Of the 60% that did not clear after 2 hours of circulation, a slower decline in HOD RBC survival was observed.

Based upon the above data, we hypothesized that the surviving RBC population had a lower susceptibility to clearance and were therefore more resistant to antibody binding. However, several alternate explanations were equally consistent with the data: 1) the incompatible HOD RBC survival was due to insufficient administration of antibody during passive immunization and 2) HOD RBC survival was due to an overloading of the phagocytic capacity within the reticuloendothelial system. To test these hypotheses, a third RBC population labeled with different dye was used. Two days after the initial incompatible transfusion,

recipient mice were exsanguinated and the blood was mixed with freshly isolated HOD blood ("fresh") labeled with DiD (see figure 4.1B for experimental design). This mixture of three RBC populations was then transfused into new C57BL/6 recipients passively immunized with the same concentration of anti-Fy^3 antibodies. No additional clearance was observed in experienced HOD RBCs compared to co-transfused FVB RBCs (Figure 4.1E). In contrast, the fresh HOD RBCs showed approximately 60% clearance, similar to the clearance observed in the 1st transfusion (Figure 4.1F). When comparing fresh and experienced HOD blood survival, fresh HOD RBCs cleared to a greater extent than experienced HOD RBCs. Because the fresh and experienced RBCs were in the same animal, and the fresh RBCs underwent 60% clearance, these findings exclude hypotheses in which experienced HOD RBCs survive due to diminished recipient capacity of clearing incompatible red cells.

Characterization of Antigen Density and Antibody Binding to Resistant cells

An alternate hypothesis for the observed incompatible RBC survival is HOD RBC dye-transfer onto non-red cells or onto host RBCs. We first sought to determine if these DiI+ cells were indeed RBCs by staining for a RBC marker. Using the gating strategy described in figure 4.1C, all DiI+ cells were found to be positive for the TER119 antigen. These data were not due to non-specific binding of anti-TER119 antibody, as DiI+ cells stained with an isotype-matched control were negative (Figure 4.2A). Post transfusion samples were also stained for the presence of the HOD antigen (specifically the Fy^3 epitope). A tight positive population was observed in non-passively immunized animals 2 hours after the 1st transfusion. In animals treated with anti-Fy^3 (MIMA29), a large positive peak was observed, however, a left shifted population appeared with a more pronounced negative peak (Figure 4.2B). Overtime, the large positive peak in anti-Fy^3 animals shifted closer to the DiO+ FVB RBCs in addition to a growing negative population. Since these populations are analyzed as % maximum, the growing negative population is most likely due to the decreasing numbers of HOD+ RBCs during

incompatible RBC removal. After 2 days of circulation, approximately half of the surviving DiI+ RBCs still remain positive for the Fy^3 epitope (Figure 4.2B). It is unlikely that the growing negative population is due to dye transfer onto cells negative for the epitope because the negative population in PBS treated mice is unchanging during the 3 day time course (Figure 4.2B). We therefore conclude that the DiI+ cells are RBCs and that about half of the DiI+ RBCs still remain HOD positive 2 days after the 1^{st} transfusion. To test if the survival of DiI+ RBCs was due to lack of antibody binding, samples were stained with anti-mouse IgGs alone (direct antiglobulin test (DAT)). Compared to control FVB RBCs, HOD RBCs were DAT+, indicating surface bound immunoglobulin (Figure 4.2C). DATs matched antigen-staining intensity throughout the time course, indicating that Fy^3 epitopes are saturated with bound antibodies throughout the 3-day course of the experiment (Figure 4.2B and 4.2C).

These results suggest that half of the HOD RBCs transfused into incompatible C57BL/6 recipients continued to circulate with detectable Fy^3 epitopes and bound antibodies up to at to two days post incompatible transfusion and upon preparation for the 2^{nd} transfusion. Resistant DiI+ cells stayed TER119 positive after the 2^{nd} transfusion. A portion of these DiI+ RBCs was still positive for the Fy^3 epitope and for bound antibodies, however, at a slightly lower intensity (data not shown).

Overall, these results demonstrate that the extended survival of the incompatible HOD RBCs is not due to an overloaded spleen, insufficient antibody presence or lack of antibody binding, and is likely not an artifact of dye transfer to non-erythroid cells or to recipient RBCs. Rather, the surviving HOD RBC population is resistant to clearance, albeit with slight clearance observed overtime. After the subsequent transfusion, no additional clearance was observed post 2^{nd} transfusion over the course of 3 days.

HOD RBC resistance is not due to complement

Dr. Göran Möller made the observation that incompatible RBC resistance may be due to a factor in the serum, possibly complement, because fewer incompatible RBCs persisted *in vivo* when mice were given heat aggregated human γ-globulin.[18] In support of this observation, proteolytically active C3 convertase cleaves C3 into C3a and C3b exposing a highly reactive thioester bond. C3b can therefore associate with the RBC surface and the RBC bound antibody. Once bound, C3b can further degrade to iC3b, C3c and C3dg, each of which can bind to various complement receptors on leukocytes, however can no longer opsonize the RBC or activate the MAC complex leading to direct cell lysis. In addition, observations of RBC circulation in incompatible animals were made after an initial sequestration of these cells. This pathway is said to occur via C3b binding to complement receptors (such as CR1) and subsequent C3b cleavage (shown to be enhanced when bound to CR1) releasing the antibody-bound RBC.[19,20] We, therefore, hypothesized that C3 is required for the resistance of incompatible RBCs during incompatible transfusion.

In order to test this hypothesis HOD and FVB RBCs were co-transfused into anti-Fy^3 passively immunized C3 KO mice. The phenotype of these mice was confirmed by complement fixation assay (data no shown). Incompatible HOD RBCs cleared in C3 KO and C57BL/6 mice alike (Figure 2.3A). No increase in antigen staining intensity or DATs were observed in C3 KO mice after the 1st incompatible transfusion, suggesting that, in C57BL/6 mice, resistant HOD RBC Fy^3 epitopes and bound antibody Fc regions are not obfuscated by C3 degraded products (Figure 4.3B and 4.3C). Blood collected from the C3 KO recipients were then mixed with freshly collected HOD blood labeled with DiD and transfused into a second set of C57BL/6 recipients also passively immunized with anti-Fy^3. Survival of HOD RBCs upon second exposure to anti-Fy^3 was uninterrupted (Figure 4.4A), which was not the case for the fresh HOD RBCs (labeled with DiD), interacting with anti-Fy^3 for the first time. Fresh HOD RBCs cleared to an even

greater extend than experienced HOD RBCs despite circulating in the same mouse (Figure 4.4B).

We conclude from these data that C3 degraded products are not required for HOD RBC clearance or resistance during an incompatible transfusion. Staining of HOD RBCs from C3 KO mice yielded similar intensity than HOD RBCs from C57BL/6 mice, suggesting that the Fy^3 epitope on HOD antigens as well as the Fc regions of bound antibodies were most likely not obfuscated by the C3 degraded products previously observed in C57BL/6 recipients of the 1st transfusion.

Resistance of HOD RBCs is due to an innate trait and is not acquired during the course of an incompatible transfusion

As previously established, MIMA29 mediated HOD RBC clearance requires Fcγ receptors (see dissertation chapter 2). In order to test if the resistance of HOD RBCs is innate or acquired during the course of an incompatible transfusion, HOD RBCs were first transfused into Fcγ KO mice with anti-Fy^3 (MIMA29) and were subsequently transfused into incompatible C57BL/6 mice. If HOD RBC resistance is acquired, incompatible transfusions into Fcγ KO mice, which do not have the ability to clear HOD RBCs, would thus yield a larger resistant HOD RBC population. Blood was collected during the course of 3 days and no HOD RBC clearance was observed during the 1st transfusion (Figure 4.5A). HOD antigen stains of DiI+ cells from PBS treated animals were identical to those from MIMA29 treated animals, with no left shift observed (Figure 4.5B). In addition, there was no difference between antigen stains and DATs of HOD RBCs in incompatible animals, indicating that RBCs were saturated with bound antibody (Figure 4.5C). Thus, stains from these collected samples showed no reduced antigen expression (Figure 4.5B) or bound-antibody (DAT) (Figure 4.5C) on the surface of incompatible DiI-labeled HOD RBCs, which contrasts the above staining results within C3KO and C57BL/6 recipients.

Once mice of the 1st transfusion were exsanguinated 2 days post transfusion, blood was combined with freshly collected HOD blood labeled with a third dye, DiD (as previously described). The mixture was then transfused into C57BL/6 recipients (2nd transfusion). Wild type mice passively immunized with anti-Fy^3 cleared "experienced" HOD RBCs from MIMA29 treated FcγR KO mice as rapidly and to the same extent as "experienced" HOD RBCs that were previously transfused into PBS treated FcγR KO mice (Figure 4.6A). Clearance of fresh HOD RBCs occurred as previously observed in C57BL/6 mice, upon first encounter with anti-Fy^3, which mimicked 1st incompatible transfusion in Figure 4.5A (Figure 4.6B). Post-2nd transfusion anti-HOD antigen and DATs stains revealed that MIMA29 exposed RBCs were bound with saturating levels of antibody (Figure 4.6C). Upon retransfusion of HOD RBCs from FcγRKO, a middle peak appeared. The highest peak in C57BL/6 mice seems to be no higher than the middle peak in FcγRKO mice (Figure 4.6C).

We conclude from these results that non-cleared incompatible HOD RBCs in FcγR KO mice (1st transfusion) clear during a 2nd transfusion in wild type mice with no noticeable increase in a HOD resistant population size. This observation suggests that resistance is not acquired during the course of an incompatible transfusion but is instead innate to the surviving HOD population.

Discussion

Presently, patients are cross-matched before every transfusion.[21] However, cases of incompatible transfusions still arise.[2,22-24] Patients with a chronic need for transfusions are at a particular risk for incompatible transfusions due to the higher rate of alloimmunization due to the more frequent exposure to new antigens[25-33] If no compatible blood is available, the patients are transfused the 'least incompatible' RBC units, which are given slowly while the patient is closely monitored for an HTR.[34] Even though HTRs can be very severe and life threatening, they do not always occur.[1,8] Understanding the intricacies of these observations will lower the risks involved with the use of incompatible RBC units.

In this current study we report the survival of HOD RBCs in incompatible animals. This survival is not due to reticuloendothelial system oversaturation, complete loss of antigen or lack of antibody binding. Even though antigen levels on incompatible HOD RBCs decreased overtime, a positive population still remained 2 days post transfusion. The decrease in HOD expression was gradual with a simultaneously growing negative population. This data can be interpreted as partial and complete antigen loss, dye transfer or both. The increase in negative population observed could be due to the relative decrease in HOD+ RBCs as they are gradually removed from circulation. As shown during the 1st transfusion survival data, 40% of incompatible HOD RBCs are rapidly cleared within 2 hours. The decrease in the rate of clearance could be due to the changing ratios of HOD positive to HOD negative RBCs. Ongoing studies are testing this interpretation.

A positive population of HOD RBCs still remained after an incompatible transfusion, which could be due to saturation of the reticuloendothelial system. The subsequent transfusion revealed that this was not the case because no additional clearance was observed in antibody experienced HOD RBCs in a second mouse. We also tested the HOD RBC's ability to resist an incompatible transfusion after circulating in a C3KO mouse because of previous research suggesting that complement is responsible for RBC resistance.[18,35] Incompatible HOD RBCs did

not clear any further after circulating in C3KO animals demonstrating a lack of C3-requirement for resistance. Consistent with these findings is the interpretation that incompatible RBCs are able to acquire resistance rapidly during the 2nd transfusion in a C57BL/6 animal.

In addressing this alternate interpretation, we asked whether resistance was acquired or innate to the RBC. Previous studies in the hGPA system (see dissertation chapters 2 and 3) did not allow us to ask this question due to the nature of the clearance. Since HOD RBCs require the presence of the common-γ chain for clearance, we therefore transfused FcγR KO mice to determine whether resistance can be acquired. After circulating in the presence of MIMA29 without clearing in FcγRKO animals, HOD RBCs cleared to previously observed levels when faced with a second wave of MIMA29. These data indicate that resistance is not acquired but is instead innate to the RBC, which is consistent with the C3KO 2nd transfusion survival data. We cannot, however rule out the possibility that knocking out the common-γ chain has changed the biology of FcγRKO mice such that resistance is no longer inducible and/or resistance is acquired in an Fcγ-dependent manner.

Antigen staining and DATs of incompatible HOD RBCs (as compared to compatible HOD RBCs) revealed a growing negative population as well as a left shift in the positive population indicating either antigen loss/obfuscation or a shift in the ratios of positive HOD and negative HOD RBC populations. This was not the case when incompatible HOD RBCs were allowed to circulated in FcγRKO mice, samples from which showed no differences in antigen staining or DAT between PBS and MIMA29 treated recipients. The lack of interaction of antibody covered HOD RBCs with Fcγ receptors could explain the continued circulation of high expressing red cells during incompatible transfusion.

The stains after a 2nd transfusion revealed the existence of a high and middle expressing HOD populations after circulating in an FcγR KO mouse. HOD RBCs that have previously circulated in C57BL/6 animals did not have high expressing RBCs. In both cases, these RBCs

were saturated with antibodies. The HOD antigen was therefore not obfuscated, thus Fc interaction with Fc receptors is possible in PBS and MIMA29 treated C57BL/6 during a 2nd transfusion. This interaction and release may be responsible for the changes in HOD expression on the surface of RBCs within C57BL/6 and C3KO recipients.

After circulating in C57BL/6 mice, HOD RBCs no longer have the high expressing HOD RBCs also suggesting that these RBCs were removed. The differences in these stains suggest a changing circulating dynamic of populations with varying antigen expression levels possibly influencing their survival rates. Ongoing studies are uncovering how expression levels effects the survival of these incompatible HOD RBCs.

Authorship and Conflict of Interest Statement: JSL performed the studies. JSL and JCZ, conceived of the studies, designed experiments, interpreted data, and contributed to the writing of the manuscript. No authors have a conflict of interest to declare.

References

1. Reid ME, Lomas-Francis C. The Blood Group Antigen FactsBook. London: Academic Press, 1997.
2. Linden JV, Paul B, Dressler KP. A report of 104 transfusion errors in New York State. Transfusion 1992;**32**: 601-6.
3. Lostumbo MM, Holland PV, Schmidt PJ. Isoimmunization after multiple transfusions. N Engl J Med 1966;**275**: 141-4.
4. Webb IJ, Anderson KC. Transfusion support in acute leukemias. Semin Oncol 1997;**24**: 141-6.
5. Galanello R, Origa R. Beta-thalassemia. Orphanet J Rare Dis 2010;**5**: 11.
6. Littlewood T, Mandelli F. The effects of anemia in hematologic malignancies: more than a symptom. Semin Oncol 2002;**29**: 40-4.
7. Hankins J, Hinds P, Day S, Carroll Y, Li CS, Garvie P, Wang W. Therapy preference and decision-making among patients with severe sickle cell anemia and their families. Pediatr Blood Cancer 2007;**48**: 705-10.
8. Janatpour KA, Kalmin ND, Jensen HM, Holland PV. Clinical outcomes of ABO-incompatible RBC transfusions. Am J Clin Pathol 2008;**129**: 276-81.
9. Baldwin ML, Ness PM, Barrasso C, Kickler TS, Drew H, Tsan MF, Shirey RS. In vivo studies of the long-term 51Cr red cell survival of serologically incompatible red cell units. Transfusion 1985;**25**: 34-8.
10. Nance ST, Arndt PA. Review: what to do when all RBCs are incompatible--serologic aspects. Immunohematology 2004;**20**: 147-60.
11. Mollison PL. Blood-group antibodies and red-cell destruction. Br Med J 1959;**2**: 1123-30.

12. Mollison PL. Factors determining the relative clinical importance of different blood-group antibodies. Br Med Bull 1959;**15**: 92-8.

13. Cutbush M, Mollison PL. Relation between characteristics of blood-group antibodies in vitro and associated patterns of redcell destruction in vivo. Br J Haematol 1958;**4**: 115-37.

14. Worlledge SM, Blajchman MA. The autoimmune haemolytic anaemias. Br J Haematol 1972;**23**: Suppl:61-9.

15. Borne AEvd, Engelfriet CP, Reynierse E, Beckers D, van Loghem JJ. Autoimmune haemolytic anaemia. VI. 51 Chromium survival studies in patients with different kinds of warm autoantibodies. Clin Exp Immunol 1973;**13**: 561-71.

16. Desmarets M, Cadwell CM, Peterson KR, Neades R, Zimring JC. Minor histocompatibility antigens on transfused leukoreduced units of red blood cells induce bone marrow transplant rejection in a mouse model. Blood 2009;**114**: 2315-22.

17. Schirmer DA, Song SC, Baliff JP, Harbers SO, Clynes RA, Krop-Watorek A, Halverson GR, Czerwinski M, Spitalnik SL. Mouse models of IgG- and IgM-mediated hemolysis. Blood 2007;**109**: 3099-107.

18. Moller G. Isoantibody-Induced Cellular Resistance to Immune Haemolysis in Vivo and in Vitro. Nature 1964;**202**: 357-9.

19. Fearon DT. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. Proc Natl Acad Sci U S A 1979;**76**: 5867-71.

20. Medof ME, Iida K, Mold C, Nussenzweig V. Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes. J Exp Med 1982;**156**: 1739-54.

21. Hillyer CD SB, Zimring JC, Abshire TC, editor *Transfusion Medicine and Hemostasis*. First ed. Burlington: Elsevier, 2009.

22. Schmidt PJ. The mortality from incompatible transfusion. Prog Clin Biol Res 1980;**43**: 251-61.

23. Sazama K. Reports of 355 transfusion-associated deaths: 1976 through 1985. Transfusion 1990;**30**: 583-90.

24. Linden JV, Wagner K, Voytovich AE, Sheehan J. Transfusion errors in New York State: an analysis of 10 years' experience. Transfusion 2000;**40**: 1207-13.

25. Hillyer CD SL, Ness PM, Anderson KC, Roback JD, editor *Blood Banking and Transfusion Medicine*. Second ed. Philadelphia: Churchill Livingstone, 2007.

26. Azarkeivan A, Ansari S, Ahmadi MH, Hajibeigy B, Maghsudlu M, Nasizadeh S, Shaigan M, Toolabi A, Salahmand M. Blood transfusion and alloimmunization in patients with thalassemia: multicenter study. Pediatr Hematol Oncol 2011;**28**: 479-85.

27. Wayne AS, Kevy SV, Nathan DG. Transfusion management of sickle cell disease. Blood 1993;**81**: 1109-23.

28. Orlina AR, Unger PJ, Koshy M. Post-transfusion alloimmunization in patients with sickle cell disease. Am J Hematol 1978;**5**: 101-6.

29. Davies SC, McWilliam AC, Hewitt PE, Devenish A, Brozovic M. Red cell alloimmunization in sickle cell disease. Br J Haematol 1986;**63**: 241-5.

30. Ambruso DR, Githens JH, Alcorn R, Dixon DJ, Brown LJ, Vaughn WM, Hays T. Experience with donors matched for minor blood group antigens in patients with sickle cell anemia who are receiving chronic transfusion therapy. Transfusion 1987;**27**: 94-8.

31. Cox JV, Steane E, Cunningham G, Frenkel EP. Risk of alloimmunization and delayed hemolytic transfusion reactions in patients with sickle cell disease. Arch Intern Med 1988;**148**: 2485-9.

32. Rosse WF, Gallagher D, Kinney TR, Castro O, Dosik H, Moohr J, Wang W, Levy PS. Transfusion and alloimmunization in sickle cell disease. The Cooperative Study of Sickle Cell Disease. Blood 1990;**76**: 1431-7.

33. Vichinsky EP, Earles A, Johnson RA, Hoag MS, Williams A, Lubin B. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. N Engl J Med 1990;**322**: 1617-21.

34. Petz LD. "Least incompatible" units for transfusion in autoimmune hemolytic anemia: should we eliminate this meaningless term? A commentary for clinicians and transfusion medicine professionals. Transfusion 2003;**43**: 1503-7.

35. Brown DL, Lachmann PJ, Dacie JV. The in vivo behaviour of complement-coated red cells: studies in C6-deficient, C3-depleted and normal rabbits. Clin Exp Immunol 1970;**7**: 401-21.

Figure legends

Figure 4.1 – Resistance of HOD incompatible RBCs in C57BL/6 mice

(A) Representative drawing of the HOD antigen and anti-Fy^3 binding. **(B)** Schematic of HOD RBC incompatible transfusion studies. **(C)** Gating strategy used during post transfusion RBC analysis with representative flow cytometric plots. **(D)** 1st transfusion: Wild type mice were passively immunized with anti- Fy^3 monoclonal antibodies (square, solid line) or control mice received PBS alone (diamond, dashed line). All recipients were then transfused with a mixture of DiI-labeled HOD RBCs and DiO-labeled FVB wild type RBCs. Percent survival of HOD RBCs was normalized to HOD RBC survival in PBS treated animals. Graph includes combined data from 3 independent experiments, each with 5-6 mice per group. **(E)** 2nd transfusion of DiI-HOD RBCs: transfused recipients were exsanguinated and blood was re-transfused into new C57BL/6 mice along with DiD-labeled HOD RBCs. DiI-HOD RBCs from PBS treated animals were transfused into PBS treated C57BL/6 mice (diamond, dashed line). DiI-HOD RBCs from PBS treated animals were transfused into anti-Fy^3 treated C57BL/6 mice (square, solid line). DiI-HOD RBCs from anti-Fy^3 treated animals were transfused into anti-Fy^3 treated C57BL/6 mice (triangle, solid line). Graphs include combined data from 3 independent experiments, each with 3 mice per group. **(F)** Fresh DiD-HOD RBCs were mixed with blood from recipients of the 1st transfusion treated with PBS (diamond, dashed line) or anti-Fy^3 (square, solid line) and transfused into PBS treated C57BL/6 mice. Fresh DiD-HOD RBCs were mixed with blood from recipients of the 1st transfusion treated with anti-Fy^3 and transfused into anti-Fy^3 treated C57BL/6 mice (triangle, solid line). Graphs include combined data from 3 independent experiments, each with 3 mice per group.

Figure 4.2 - Stains of HOD incompatible RBCs after 1st transfusion in C57BL/6 mice

(A) Histogram represents staining with anti-Ter119 (black line) or isotype matched control (shaded gray) of post 1st transfusion DiI-HOD (Experienced) RBCs. **(B)** Histograms represent

staining with anti-Fy^3 (MIMA29) monoclonal antibodies and secondary anti-mouse IgGs of DiI-labeled HOD RBCs (black line) or DiO-labeled control wild type FVB RBCs (shaded gray) after circulation in PBS or MIMA29 treated C57BL/6 mice 2 hours, 1 day and 2 days post 1st transfusion. **(C)** Histograms represent staining with secondary anti-mouse IgGs alone (DAT) of DiI-labeled HOD RBCs (black line) or DiO-labeled control wild type FVB RBCs (shaded gray) after circulation in PBS or MIMA29 treated C57BL/6 mice 2 hours, 1 day and 2 days post 1st transfusion.

Figure 4.3 – Clearance and staining of HOD incompatible RBCs in C3 KO mice (1st transfusion)

(A) C57BL/6 (solid lines) and C3 KO (dashed lines) mice were passively immunized with anti-Fy^3 monoclonal antibodies (square) or control mice received PBS alone (triangle). All recipients were then transfused with a mixture of DiI-labeled HOD RBCs and DiO-labeled FVB wild type RBCs. Percent survival of HOD RBCs was normalized to HOD RBC survival in PBS treated animals. Graph includes combined data from 3 independent experiments, each with 5-6 mice per group. **(B)** Histograms represent staining with anti-Fy^3 (MIMA29) monoclonal antibodies and secondary anti-mouse IgGs of DiI-labeled HOD RBCs (black line) or DiO-labeled control wild type FVB RBCs (shaded gray) after circulation in PBS or MIMA29 treated C3 KO mice 2 hours, 1 day and 2 days post 1st transfusion. **(C)** Histograms represent staining with secondary anti-mouse IgGs alone (DAT) of DiI-labeled HOD RBCs (black line) or DiO-labeled control wild type FVB RBCs (shaded gray) after circulation in PBS or MIMA29 treated C3 KO mice 2 hours, 1 day and 2 days post 1st transfusion.

Figure 4.4 - Continued resistance of incompatible HOD RBCs from C3 KO mice in C57BL/6 mice (2nd transfusion)

(A) DiI-HOD (Experienced) RBCs from PBS treated C3 KO animals were transfused into PBS treated C57BL/6 mice (diamond, dashed line). DiI-HOD RBCs from PBS treated C3 KO animals were transfused into anti-Fy^3 treated C57BL/6 mice (square, solid line). DiI-HOD RBCs from anti-Fy^3 treated C3 KO animals were transfused into anti-Fy^3 treated C57BL/6 mice (triangle, solid line). Graphs include combined data from 3 independent experiments, each with 3 mice per group. **(B)** Fresh DiD-HOD RBCs were mixed with blood from C3 KO recipients of the 1st transfusion treated with PBS (diamond, dashed line) or anti-Fy^3 (square, solid line) and transfused into PBS treated C57BL/6 mice. Fresh DiD-HOD RBCs were mixed with blood from C3 KO recipients of the 1st transfusion treated with anti-Fy^3 and transfused into anti-Fy^3 treated C57BL/6 mice (triangle, solid line). Graphs include combined data from 2 independent experiments, each with 3 mice per group.

Figure 4.5 – Lack of incompatible HOD RBC clearance in Fcγ receptor KO mice and staining of HOD incompatible RBCs (1st transfusion)

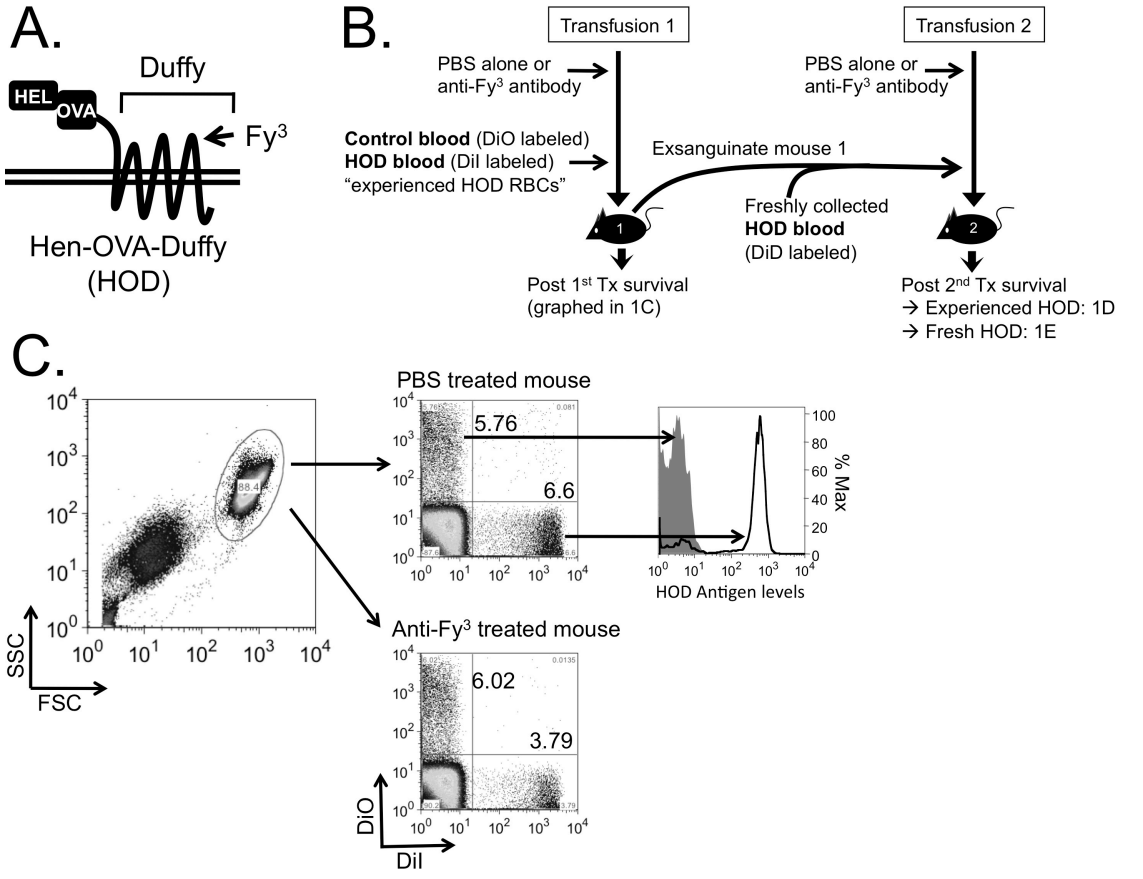
(A) C57BL/6 (solid lines) and FcγR KO (dashed lines) mice were passively immunized with anti-Fy^3 monoclonal antibodies (square) or control mice received PBS alone (triangle). All recipients were then transfused with a mixture of DiI-labeled HOD RBCs and DiO-labeled FVB wild type RBCs. Percent survival of HOD RBCs was normalized to HOD RBC survival in PBS treated animals. Graph includes combined data from 3 independent experiments, each with 5-6 mice per group. **(B)** Histograms represent staining with anti-Fy^3 (MIMA29) monoclonal antibodies and secondary anti-mouse IgGs of DiI-labeled HOD RBCs (black line) or DiO-labeled control wild type FVB RBCs (shaded gray) after circulation in PBS or MIMA29 treated FcγR KO mice 2 hours, 1 day and 2 days post 1st transfusion. **(C)** Histograms represent staining with secondary anti-mouse IgGs alone (DAT) of DiI-labeled HOD RBCs (black line) or DiO-labeled control wild

type FVB RBCs (shaded gray) after circulation in PBS or MIMA29 treated FcγR KO mice 2 hours, 1 day and 2 days post 1st transfusion.

Figure 4.6 – Clearance of experienced incompatible HOD RBCs, from FcγR KO mice, in C57BL/6 mice (2nd transfusion)

(A) DiI-HOD (Experienced) RBCs from PBS treated FcγR KO animals were transfused into PBS treated C57BL/6 mice (diamond, dashed line). DiI-HOD RBCs from PBS treated FcγR KO animals were transfused into anti-Fy^3 treated C57BL/6 mice (square, solid line). DiI-HOD RBCs from anti-Fy^3 treated FcγR KO animals were transfused into anti-Fy^3 treated C57BL/6 mice (triangle, solid line). Graphs include combined data from 3 independent experiments, each with 3 mice per group. **(B)** Fresh DiD-HOD RBCs were mixed with blood from FcγR KO recipients of the 1st transfusion treated with PBS (diamond, dashed line) or anti-Fy^3 (square, solid line) and transfused into PBS treated C57BL/6 mice. Fresh DiD-HOD RBCs were mixed with blood from FcγR KO recipients of the 1st transfusion treated with anti-Fy^3 and transfused into anti-Fy^3 treated C57BL/6 mice (triangle, solid line). Graphs include combined data from 3 independent experiments, each with 3 mice per group. **(C)** Representative histograms of staining with anti-Fy^3 (MIMA29) monoclonal antibodies and secondary anti-mouse IgGs or with secondary anti-mouse IgGs alone (DAT) of samples collected 2 hours post 2nd transfusion. DiI-labeled HOD RBCs (black line) or DiO-labeled control wild type FVB RBCs (shaded gray) were transfused into MIMA29 treated C57BL/6 or FcγR KO mice (1st transfusion) and were subsequently transfused into PBS or MIMA29 treated C57BL/6 mice (2nd transfusion). Graphs include combined data from 2 independent experiments, each with 3 mice per group.

Figure 4.1


A.
Duffy
HEL
OVA
Fy3
Hen-OVA-Duffy
(HOD)
B.
Transfusion 1
Transfusion 2
PBS alone or
anti-Fy3 antibody
PBS alone or
anti-Fy3 antibody
Control blood (DiO labeled)
HOD blood (DiI labeled)
"experienced HOD RBCs"
Exsanguinate mouse 1
Freshly collected
HOD blood
(DiD labeled)
Post 1st Tx survival
(graphed in 1C)
Post 2nd Tx survival
→ Experienced HOD: 1D
→ Fresh HOD: 1E
C.
PBS treated mouse
5.76
6.6
% Max
HOD Antigen levels
Anti-Fy3 treated mouse
6.02
3.79
SSC
FSC
DiO
DiI

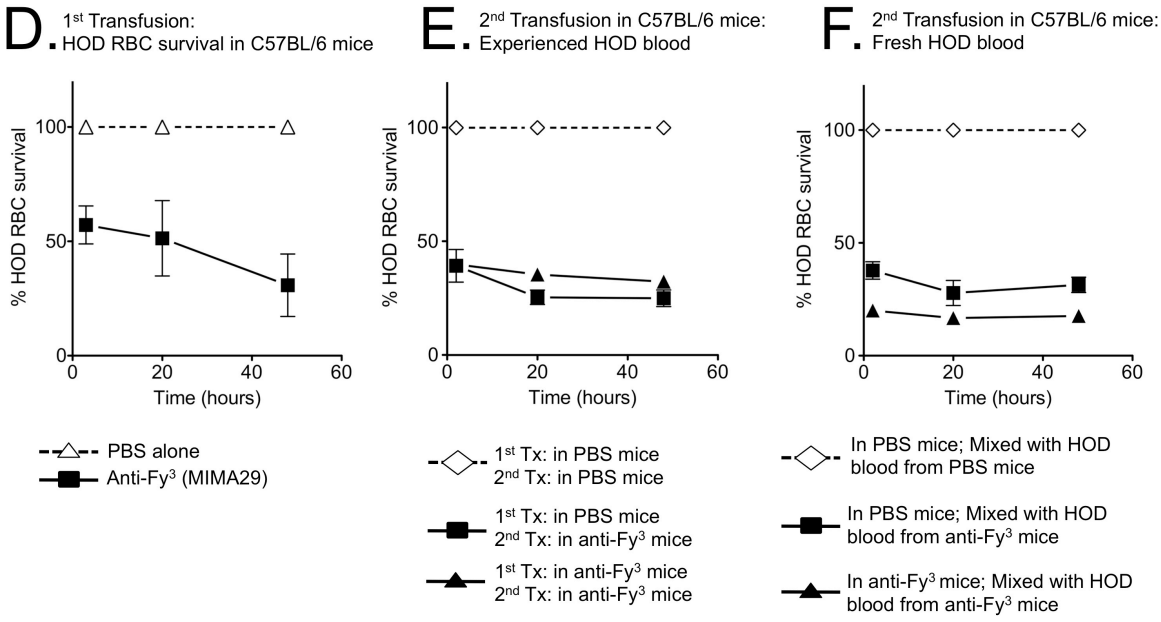

D. 1st Transfusion:
HOD RBC survival in C57BL/6 mice
E. 2nd Transfusion in C57BL/6 mice:
Experienced HOD blood
F. 2nd Transfusion in C57BL/6 mice:
Fresh HOD blood
% HOD RBC survival
Time (hours)
PBS alone
Anti-Fy3 (MIMA29)
1st Tx: in PBS mice
2nd Tx: in PBS mice
1st Tx: in PBS mice
2nd Tx: in anti-Fy3 mice
1st Tx: in anti-Fy3 mice
2nd Tx: in anti-Fy3 mice
In PBS mice; Mixed with HOD
blood from PBS mice
In PBS mice; Mixed with HOD
blood from anti-Fy3 mice
In anti-Fy3 mice; Mixed with HOD
blood from anti-Fy3 mice

Figure 4.2

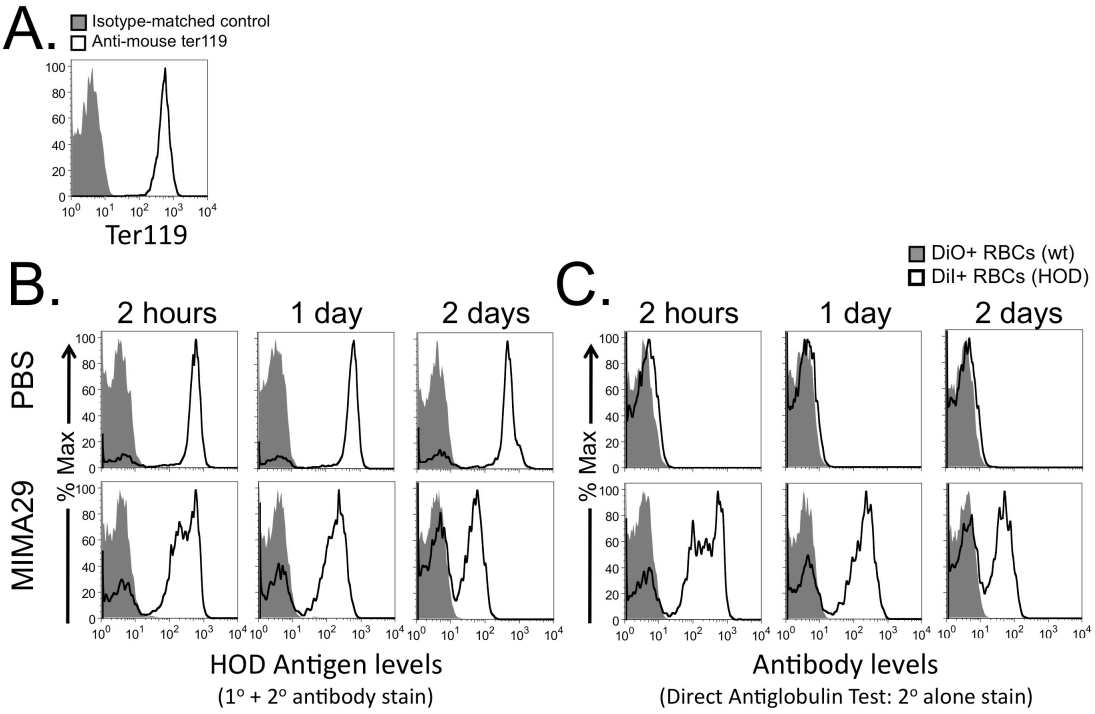

A.
Isotype-matched control
Anti-mouse ter119
Ter119
B.
C.
DiO+ RBCs (wt)
DiI+ RBCs (HOD)
2 hours
1 day
2 days
PBS
MIMA29
% Max
HOD Antigen levels
(1° + 2° antibody stain)
Antibody levels
(Direct Antiglobulin Test: 2° alone stain)

Figure 4.3

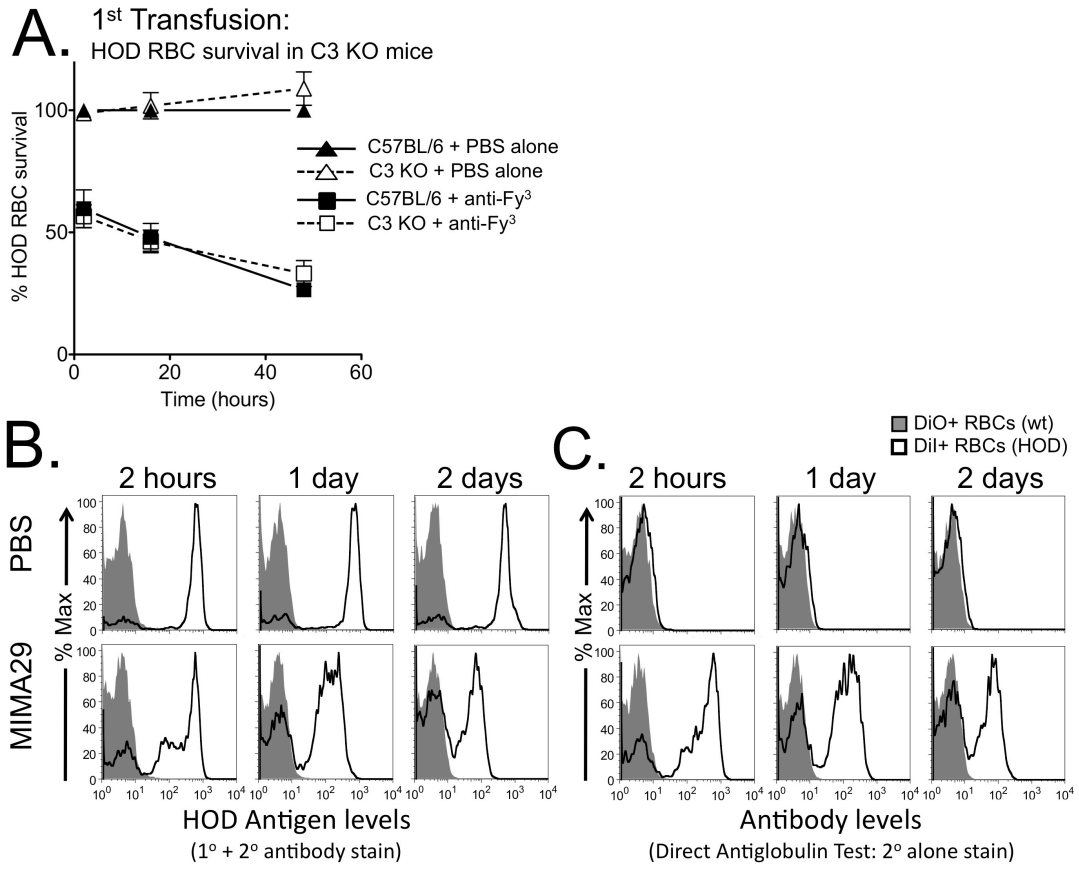

A.
1st Transfusion:
HOD RBC survival in C3 KO mice
% HOD RBC survival
100
50
0
0
20
40
60
Time (hours)
C57BL/6 + PBS alone
C3 KO + PBS alone
C57BL/6 + anti-Fy3
C3 KO + anti-Fy3
B.
2 hours
1 day
2 days
PBS
MIMA29
% Max
HOD Antigen levels
(1° + 2° antibody stain)
C.
DiO+ RBCs (wt)
DiI+ RBCs (HOD)
2 hours
1 day
2 days
% Max
Antibody levels
(Direct Antiglobulin Test: 2° alone stain)

Figure 4.4

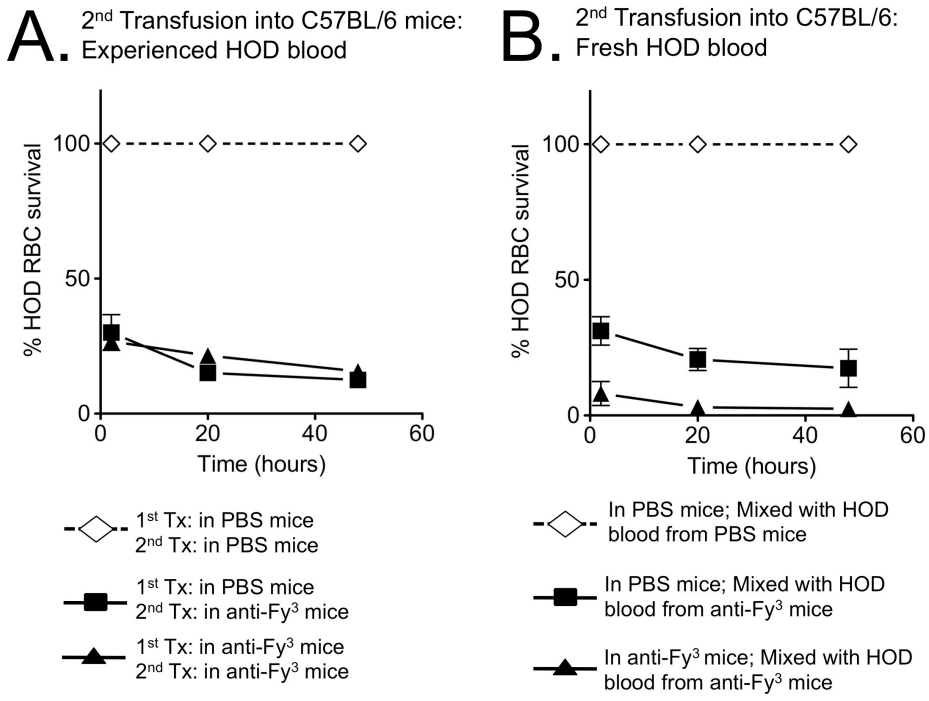

A.
2nd Transfusion into C57BL/6 mice:
Experienced HOD blood
% HOD RBC survival
100
50
0
0
20
40
60
Time (hours)
1st Tx: in PBS mice
2nd Tx: in PBS mice
1st Tx: in PBS mice
2nd Tx: in anti-Fy3 mice
1st Tx: in anti-Fy3 mice
2nd Tx: in anti-Fy3 mice
B.
2nd Transfusion into C57BL/6:
Fresh HOD blood
% HOD RBC survival
100
50
0
0
20
40
60
Time (hours)
In PBS mice; Mixed with HOD
blood from PBS mice
In PBS mice; Mixed with HOD
blood from anti-Fy3 mice
In anti-Fy3 mice; Mixed with HOD
blood from anti-Fy3 mice

Figure 4.5

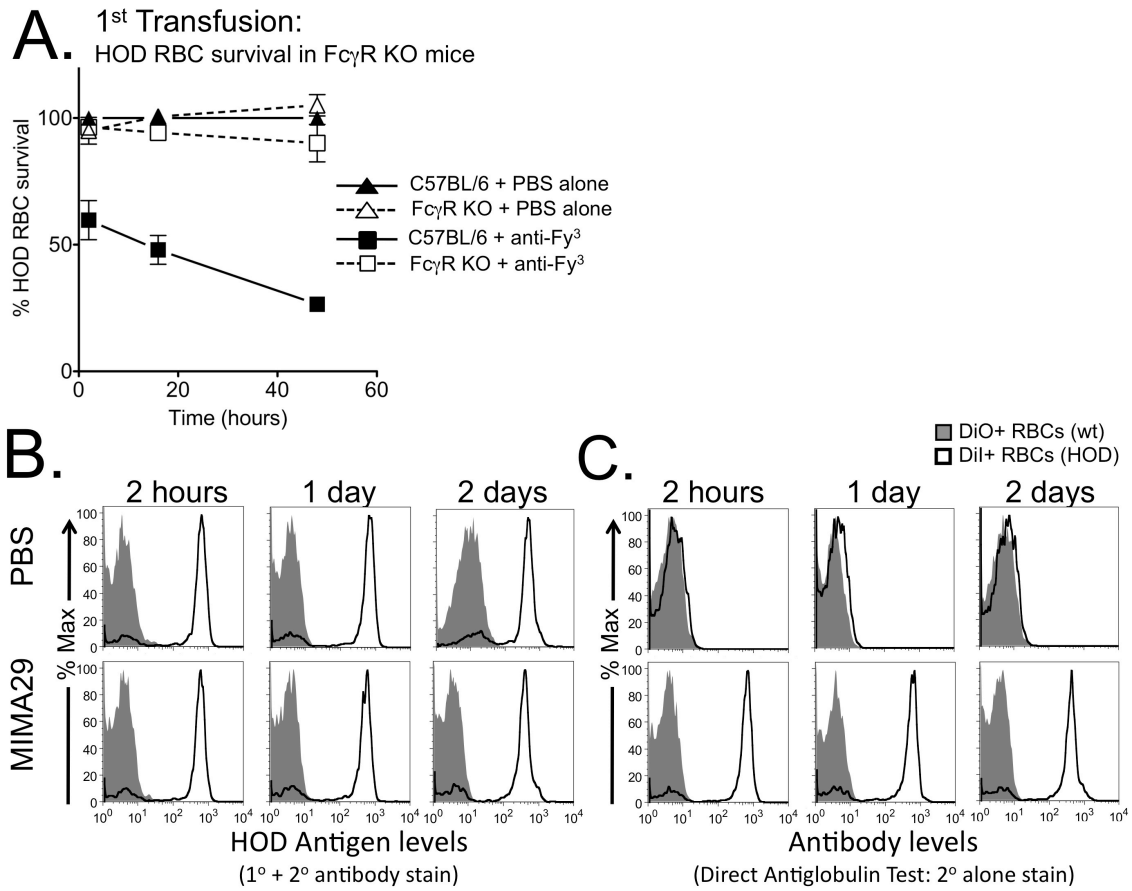
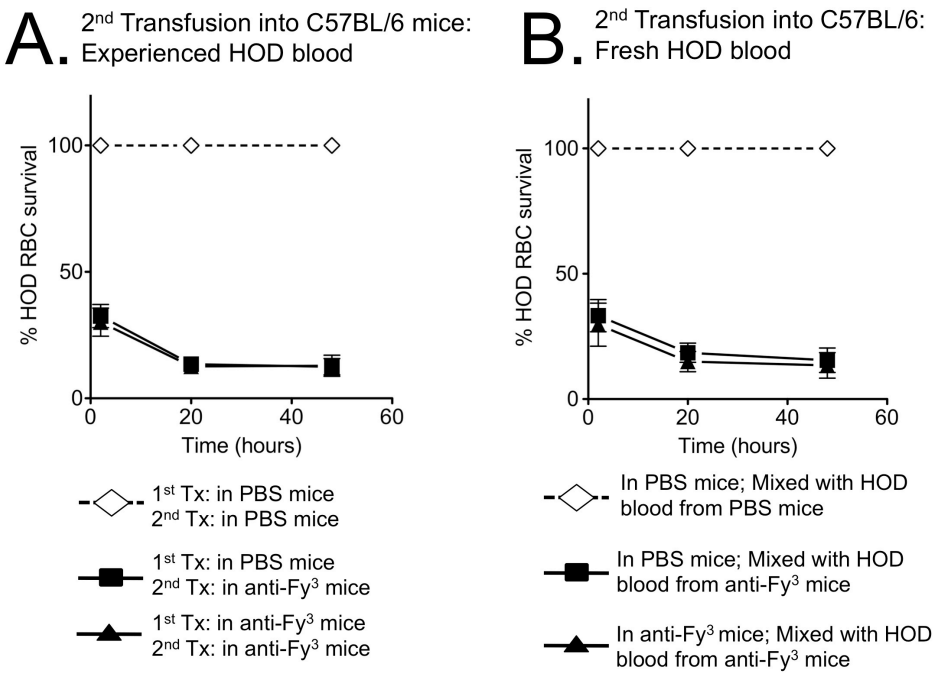

A.
1st Transfusion:
HOD RBC survival in FcγR KO mice
% HOD RBC survival
100
50
0
0
20
40
60
Time (hours)
C57BL/6 + PBS alone
FcγR KO + PBS alone
C57BL/6 + anti-Fy3
FcγR KO + anti-Fy3
B.
2 hours
1 day
2 days
PBS
MIMA29
% Max
HOD Antigen levels
(1° + 2° antibody stain)
C.
DiO+ RBCs (wt)
DiI+ RBCs (HOD)
2 hours
1 day
2 days
% Max
Antibody levels
(Direct Antiglobulin Test: 2° alone stain)

Figure 4.6


A.
2nd Transfusion into C57BL/6 mice:
Experienced HOD blood
% HOD RBC survival
100
50
0
0
20
40
60
Time (hours)
1st Tx: in PBS mice
2nd Tx: in PBS mice
1st Tx: in PBS mice
2nd Tx: in anti-Fy3 mice
1st Tx: in anti-Fy3 mice
2nd Tx: in anti-Fy3 mice
B.
2nd Transfusion into C57BL/6:
Fresh HOD blood
% HOD RBC survival
100
50
0
0
20
40
60
Time (hours)
In PBS mice; Mixed with HOD
blood from PBS mice
In PBS mice; Mixed with HOD
blood from anti-Fy3 mice
In anti-Fy3 mice; Mixed with HOD
blood from anti-Fy3 mice

Chapter 5

Discusion

Summary

Transfusion therapy is widely used to treat acute and chronic anemia. According to the FDA, about 24 million components were transfused in the US in 2008, highlighting the importance of transfusion therapy.[1] Although compatibility testing has been used to avoid pathophysiologies such as hemolytic transfusion reactions (HTRs), incompatible transfusions still occur. In the 2010 fiscal year, 64 transfusion-related fatalities were reported to the FDA.[1] As previously discussed, incompatible transfusions can go un-noticed and therefore are not always reported. Within perceived incompatible transfusions, some do not lead to pathophysiology and the survival of transfused RBCs is normal. Therefore, in rare instances some physicians may give incompatible blood if no compatible blood is available and the risk of morbidity and mortality during incompatible transfusions outweighs negative outcomes from not transfusing critically anemic patients.

In this thesis, the human blood group glycophorin A (hGPA) and Duffy (part of HOD) antigens were used to study incompatible transfusions in mice. Antibodies against the human blood group Duffy (as part of a fusion protein HOD) were found to clear via Fc-receptors (one of the 2 established pathways of RBC clearance). In contrast, antibodies to hGPA were found to not employ neither complement nor Fc receptors, but instead use a third unique biphasic mechanism involving sequestration via RBC aggregation and phagocytosis. In the first phase, anti-hGPA antibodies agglutinate hGPA RBC, sequestering them from circulation. During the second phase, phagocytic cells remove the sequestered RBCs in an Fc-receptor independent manner. A cytokine burst was detected during the first phase of RBC removal, which was found to require Fc-receptors. These findings demonstrate the existence of a new pathway of RBC removal, the involvement of 2 phases of clearance and a decoupling of clearance and cytokine release.

In knowing the clearance pathways of RBCs bearing these 2 antigens, the RBC's ability to survive *in vivo* or resist against incompatible transfusions was investigated. hGPA and HOD RBCs were found to not completely clear when faced with a bolus of anti-hGPA and anti-Duffy antibodies, respectively. During both hGPA and HOD incompatible transfusions, a population of RBCs was found to be resistant. Previous findings suggest that complement is required for resistance and others have hypothesized C3-mediated sequestration and release of incompatible RBCs. Since neither anti-hGPA nor anti-Duffy were found to use C3 for clearance, C3 was investigated as a possible mechanism involved in resistance. Resistance of hGPA and HOD RBCs were found to not require C3. These findings were particularly interesting for the hGPA antigen since sequestration was found to be involved in the first phase of clearance. Titrations of anti-hGPA antibody mediated clearance suggests a spectrum of RBC susceptibly among hGPA RBCs. Due to the nature of HOD RBC clearance (i.e. the inability to clear in FcγR KO mice), it was possible to test whether resistance was innate or acquired during the course of an incompatible transfusion. In the case of HOD RBCs, we concluded that resistance is innate to the circulating RBC population.

Overall, incompatible RBC clearance pathways seem to vary among blood group antigens and/or binding antibody. The resulting RBC resistance may be relative to the blood group in question. This thesis elucidates a novel RBC clearance pathway and confirms resistance as a phenomenon. In this particular system, resistance was not found to require complement as previously suggested, and resistance appears to be intrinsic to the surviving RBC population.

Discussion

Clearance of incompatible RBCs

In order to shed some light on the biology of incompatible transfusions, we developed a murine transfusion model with the use of both anti-Duffy and anti-hGPA monoclonal antibodies. hGPA (member of the MNS blood group) and Duffy (as part of a fusion protein HOD) are characterized proteins that are structurally different and potentially reflect the biology of other blood group antigens. Transgenic mice expressing the human glycophorin A antigen and the blood group Duffy (as part of HOD) were used as donors.

HOD, a fusion protein that includes the human blood group Duffy was used as a marker for "typical" RBC clearance along side of hGPA incompatible studies. HOD is a 7-membrane pass protein (Duffy) with ovalbumin and hen-egg-lysozime fused on the N-terminus. Prior to transfusions, mice were passively immunized with MIMA29, an IgG2a antibody specific for the 3rd extracellular loop on the Duffy antigen (called Fy^3). The human clinical significance of anti-Fy^3 antibodies ranges from mild to moderate or are delayed transfusion reactions and in the context of HDN, only rare mild reactions have been observed.[2] In our mouse model, anti-Fy^3 mediated clearance of HOD RBCs was dependant upon Fc-receptors. With the understanding that there are 2 major pathways of clearance employed by incompatible RBCs (via Fc-receptors or complement), we chose to include this antigen in our studies as an example of Fc-mediated clearance and as a possible reflection of previous transfusion research. It is unknown, however if Duffy RBC clearance via anti-Fy^3 is mediated by Fc receptors in humans.

GPA is a heavily glycosylated single-pass membrane protein, thought to be responsible for reducing RBC-RBC and RBC-vessel adhesion by coating the RBCs with negative charge due to the extensive glycosylation of GPA. Most humans express this antigen at high levels (800,000-1 million copies per cell).[2,3] Individuals that do not

express this antigen (Ena^-) are physiologically normal. In the context of transfusions or pregnancy, antibodies against GPA can range from eliciting no reaction to being clinically significant. The M and N variants of GPA elicit antibodies that are not clinically significant.[2] If no pathophysiology was observed during human incompatible transfusions, it can be assumed that enough of the transfused RBCs survive to alleviate symptoms of anemia. It is unknown how many (if any) GPA RBCs are cleared in a human incompatible transfusion. Human GPA RBC survival studies are needed to address this issue. In our model of incompatible transfusion, two IgG1 anti-hGPA monoclonal antibodies were used. 10F7 recognizes a non-polymorphic epitope on GPA and 6A7 binds the M variant of GPA.[4-6] These antibodies were found to clear hGPA RBCs transfused into C57BL/6 mice at a level relative to antibody concentration. When 10F7 is used at 500mg, hGPA RBCs clear to about 20%. Once titrated down to 31.3mg, 10F7 leaves about 75% incompatible hGPA RBCs in circulation. The murine hGPA incompatible transfusion may reflect human hGPA incompatible transfusion biology, however incompatible hGPA RBC survival, as a function of antibody serum levels has yet to be elucidated in humans. In addition, our studies did not measure serum levels of monoclonal antibodies after passive immunization. The body distribution and half-life of injected antibody could further explain the kinetics of hGPA RBC survival (as well as HOD RBC survival).

In studying the similarities and differences between 2 blood group antigens, we were able learn more about the biology of incompatible RBC clearance. With the transfusion of both hGPA RBCs and HOD RBCs, elevated serum cytokine levels were observed in our mouse model, which would suggest a transfusion reaction. Cytokine secretions in response to these transfusions were dependent upon the presence of Fcγ receptor common-γ chain. In the case of HOD RBCs, clearance and cytokine secretion both required functional Fcγ receptors. However, hGPA RBC clearance did not require

Fcγ receptors while cytokine secretion did. The decoupling of RBC removal and cytokine secretion is a novel concept, which needs further exploration.

Therapeutic drugs targeting complement (such as anti-C5 antibodies known as eculizumab) or Fc receptors (such as IVIG) may not be useful against the clearance of RBCs expressing certain blood groups or when particular antibody isotypes are present in the incompatible recipient.[3,7] Patients with chronic anemia are prime candidates for drugs that diminish RBC clearance. Splenectomies have also been used as a means of reducing transfusion dependency. These immune regulatory therapies make the patient susceptible to infections and even have conflicting results with regards to patient hematocrit.[8] It is therefore important to understand antibody-mediated RBC clearance in order to customize therapeutic intervention, which can enhance the efficacy of the treatment while minimizing side effects.

This dissertation suggests that liposome-encapsulated bisphosphonates can extend the survival of incompatible RBCs. This treatment would have an effect on red cells utilizing a phagocyte-dependent clearance, which can be Fc-dependent or Fc-independent (but phagocyte dependent). Clodronate has already undergone clinical trials to treat bone loss in women with breast cancer.[9] This treatment temporarily removes phagocytes from the host, which would be enough time to allow an incompatible transfusion to occur. It is unclear if this treatment would also block transfusion induced cytokine bursts, however. Careful studies would need to be done prior to using this therapy in humans, including considerations of both efficacy and toxicity.

The current studies reveal that more than 2 pathways exist for incompatible RBC clearance. It is unclear which mechanism is most common. Clearance mechanisms of RBCs expressing other blood group antigens need to be studied. It is unclear which clearance pathway is most commonly used by various antigens. It is also unclear

whether the use of these various pathways depends on the nature of the antigen or the offending antibody.

Resistance of incompatible RBCs

With the understanding that HOD RBCs clear via the Fc-mediated pathway and that hGPA RBCs clear via agglutination-mediated sequestration followed by phagocytosis, we investigated why these RBCs do not clear 100%. Previous studies had demonstrated that RBCs are capable of circulating in an incompatible host.[10-12] Further investigation implied that the complement cascade had a contradicting role of protecting incompatible RBCs from clearance.[13-15]

In both hGPA and HOD incompatible transfusion model systems, we confirmed the existence of a persistent population of incompatible RBCs. After transfusion into incompatible complement knock animals (C3 KO mice), the surviving RBC population was still able to survive in C57BL/6 animals. We therefore rejected the hypothesis that resistance is induced by complement, specifically C3. Our results cannot rule out the existence of such a pathway in other blood group settings, such as those used in previous studies. Immunological clearance of incompatible RBCs is not a uniform pathway used by all blood groups, therefore it is not unreasonable to suggest the existence of multiple resistance pathways.

Further investigation into the biology of resistance revealed that hGPA and HOD RBCs have a few differences. hGPA RBC percent survival was found to level off 2 hours post transfusion. Even though these resistant RBCs were antigen positive, a slight decrease in expression was observed overtime as compared to compatible hGPA RBCs. This could be due to either antigen obfuscation or antigen loss. A study by Zimring *et al*. showed, however, that antigen loss occurs only with the simultaneous use of monoclonal antibodies with specificities to 2 or more different epitopes. This report on antigen loss

was done using RBC surface hen-egg-lysozyme and does not exclude the possibility of antigen loss mediated by one kind of monoclonal antibody in other blood group systems.[16] In addition, the hGPA protein can form homodimers,[17] thus potentially offering 2 epitopes to circulating 10F7 anti-hGPA monoclonal antibodies. hGPA dimer antigen loss needs to be further investigated as potential alternate mechanism of antigen loss.

The loss of detectable antigen on incompatible hGPA RBCs may indicate disturbance of the RBC surface, which could be due to transient interactions with receptors and proteins. In elucidating the clearance pathway of hGPA RBCs in the presence of anti-hGPA monoclonal antibodies, the antibody itself was directly mediating the RBC removal from circulation, through agglutination. No further clearance was observed after the initial sequestration, which would suggest that phagocytes remove incompatible hGPA RBCs only once sequestered. It can, therefore, be inferred that the resistant hGPA population is escaping antibody directed agglutination. The changes observed on the incompatible hGPA surface may contribute to that escape through antigen loss or obfuscation. Figure 5 in chapter 2 showed the escape of some of these agglutinates as well as some individual DiI+ RBCs. Changes induced by these antibodies need to be further investigated.

A potential avenue to explore is the change of antigen topography within the RBC membrane. Even though these incompatible hGPA RBCs are still highly hGPA positive, the distribution of these proteins may have changed, therefore reducing the anti-hGPA antibody's ability to agglutinate these RBCs (such a change would not be detected by flow cytometry which only measures mean fluorescence). In addition to forming homodimers, hGPA is also able to form heterodimers with membrane proteins that associate with RBC cytoskeleton.[17-19] If antigen is occurring, the hGPA proteins that still remain on the surface of hGPA RBCs would most likely be those that are forming heterodimers with members of the cytoskeleton-associating complexes, which would be

more difficult to dissociate from (as oppose to GPA proteins only associated with itself). The resulting spread of hGPA proteins could be responsible for the different topography. The membrane spread of proteins has previously been studied via electron microscopy.[20-22] Sanan *et al.* have devised a protocol to study membrane spread with the use of transmission electron microscopy, which allows for the analysis of larger surface areas at greater magnification.[23] This imaging technique would be a powerful tool to study hGPA membrane distribution on post incompatible transfusion RBCs.

RBC age is another possible explanation for RBC survival in the face of binding antibodies. As RBCs age, the membrane and surface proteins change, which tend to make the cells susceptible to clearance. Chasis *et al*. demonstrated that upon hGPA binding with lectin or antibodies, including 10F7, the RBC membrane became more rigid. In addition, signal transduction was found to be a result of GPA binding.[24-27] The RBCs that continue to survive after facing a wave of antibodies may be a result of a selective pressure on circulating RBCs with the most malleable membranes. Biochemical studies are needed to investigate the characteristics of resistance, including membrane flexibility, possible signal transduction, as well as antigen differences and membrane spread.

When titrating the anti-hGPA antibody, different levels of clearance are observed. Moreover, with each anti-hGPA dose, the initial clearance was rapid and no additional RBC removal was observed. Preliminary results of experiments testing the resistance capacity of incompatible hGPA RBCs demonstrated increased susceptibility (during the 2^{nd} transfusion) with decreasing anti-hGPA dose during the 1^{st} transfusion. These experiments have recently been repeated but have yielded inconsistent results.

It is unclear whether the HOD RBCs undergo the same mechanism of resistance as hGPA RBCs. According to the data in chapter 4, HOD resistance is innate to the circulating RBCs, suggesting that anti-Fy^3 (MIMA29) eliminates susceptible RBCs

therefore selecting for the resistant HOD RBCs. Since both hGPA and HOD RBCs have populations of resistant cells, it is conceivable that both have the same mechanism of resistance. With that in mind, a potential mechanism of resistance in common with hGPA, is the different distribution of resistant surface antigens, which would diminish Fc-receptor mediated phagocytosis efficacy of certain RBCs. HOD resistance could also result from an age dependent process and/or from antibody-induced membrane rigidity (also mentioned as possible hGPA resistance mechanisms).

HOD RBC resistance may be unique to this antigen and/or to antigens utilizing the Fc-receptor mediated clearance pathway. The FcγRII Fc-receptor in mice (FcγRIIb in humans) has the ability to transduce an inhibitory signal via homoaggregation. In general, receptor-engaged antibodies need to be close together, which is most likely to occur when antigen surface is bound by a high concentration of antibody. Antibody *in vivo* titrations of MIMA29 and resulting stains revealed that HOD RBCs are saturated with bound antibodies. Thus, the HOD antigen distribution and concentration on RBCs could be a determining factor of RBC survival and resistance via FcγRII induced inhibition. These Fc receptor-antibody interactions could also explain the antigen perturbations observed overtime with antigen stains. FcγRII engagement could potential explain hGPA resistance as well. Studies on hGPA RBC clearance revealed that the common-γ chain was not required for clearance, but does not rule out the involvement of FcγRII (which does not have an associated common-γ chain) during clearance or resistance. The high level of hGPA expression (1 million copies per RBC) could facilitate FcγRII homoaggregation. In either case (HOD or hGPA), antibody titers or antigen levels could play a role in the resistance of incompatible red cells.

Future directions

It is still unclear which receptors of the phagocytes are responsible for the permanent removal of incompatible hGPA RBCs and therefore need further investigation. Blocking agents need to be tested as possible treatments against incompatible RBC removal and host reaction. The hGPA RBCs disappear quickly, although it is not known where they are sequestered and ultimately consumed. Incompatible hGPA RBCs may accumulate in the spleen although since clearance still occurs in splenectomized mice, the spleen is not the only organ involved.

Splenectomies are used with caution to treat a subset of patients who are refractory to other treatments (in particularly in AIHA). Splenectomies do not always help RBC survival; therefore studies are needed to better predict when splenectomies are most beneficial for patients in need of this invasive surgery.

Because the immunology of different blood groups is known to differ from each other, clearance mechanisms of other blood groups need to be investigated. It is unclear how often agglutination, Fc-receptor or complement mediated pathways are utilized by other antigens. Moreover, other pathways may exist in addition to these three mentioned. Understanding clearance mechanisms may also help in understanding resistance.

Some immunomodulatory treatments have already been tested in humans. Clinical trials using clodronate, IVIG and anti-complement antibodies (alone or in combination) can be used along side incompatible transfusions to transiently diminish clearance of RBCs. However, there are differences in Fc receptors as well as in their binding affinities to antibodies between mice and humans, thus observations made in mice may not necessarily reflect human biology, which also needs further investigation.

Our data on hGPA and HOD RBC mechanisms of resistance rule out some likely hypotheses, but ultimately do not reveal a mechanism. HOD resistance is innate

although it is unknown whether hGPA resistance is innate or acquired over the course of an incompatible transfusion. Other antigens would need to be tested in this regard as well, since it is unclear whether other antigens can lead to RBC resistance.

Clinically, it would be important to find out whether incompatible RBCs can be made resistant (maybe with antigens other than Duffy) or if these cells can be pre-selected prior to transfusion. Since age may be a factor, clinical trials on RBC age and related survival during incompatible transfusions would determine if certain RBC units are more appropriate for incompatible transfusions.

Antibody titers of patients could also be a determining factor for resistant RBC selection. As mentioned earlier, high titers may be responsible for allowing the extended survival of certain RBCs, additional animal studies are needed in order to determine if this is the case.

General conclusions

Although transfusion has been used for centuries, we have only just begun to understand its therapeutic potential in treating acute and chronic anemia. Compatibility testing has been used to avoid pathophysiologies such as HTRs, however incompatible transfusions still occur and are sometimes intentionally performed when no compatible blood is found in time. This dissertation sought to study the survival and clearance of transfused RBCs by studying 2 different RBC antigens (hGPA and Duffy as part of HOD).

The data presented herein have shown that a novel RBC clearance pathway exists (agglutination-mediate sequestration and phagocytosis) and that cytokine secretion is not necessarily directly linked to phagocytosis of RBCs (cytokine secretion was found to be Fc-receptor dependent whereas clearance was shown to be Fc-receptor independent). It is still unclear whether this pathway is also used by other antigens or if this is unique to the hGPA antigen expressing RBCs. Studies performed on human incompatible transfusions seldom include information about the donor RBC surface biochemistry or about the antibody isotype found in the serum of recipients. Mouse transfusion studies have only started to uncover some of those mysteries.

Even though clearance of RBCs during incompatible transfusions, or within AIHA patients, is thought to occur in one of 2 pathways (Fc-receptor mediated or complement mediated), it is not known how often these pathways are used by clearing RBCs. With the uncovering of a new clearance mechanism during incompatible transfusions, in support of data in other systems (AIHA), we added a third option for clearance, the frequency of which is unknown in human transfusions.

In addition to studying RBC clearance, RBC persistence in the presence of binding antibody was found to be a phenomenon of resistance against clearance. The mechanism of clearance resistance is still unknown, however data from our two systems

have rejected a long-standing hypothesis of resistance via complement protection. In addition, these data suggest an innate trait that would make a certain red cell population less susceptible to clearance. In humans, it is unclear why the rate of alloimmunization in transfusions is relatively low when compared to immune responses to diseases during which antibody responses are close to 100% and to solid organ transplants, which lead to near 100% rejection (without potent pharmacological intervention) due to RBC alloantigens.

Understanding these anomalies would allow physicians to customize therapeutic interventions for their patients. With the low supply of certain blood types, finding compatible blood is not always possible; therefore making incompatible blood safe for transfusions can be critical to patients with rare blood types. This dissertation is hopefully a step towards finding these answers.

References

1. Fatalities Reported to FDA Following Blood Collection and Transfusion. Annual Summary for Fiscal Year 2010. In: Services DoHaH, ed. Silver Spring: U.S. Foud and Drug Administration, 2010.
2. Reid ME, Lomas-Francis C. The Blood Group Antigen FactsBook. London: Academic Press, 1997.
3. Hillyer CD SL, Ness PM, Anderson KC, Roback JD, editor *Blood Banking and Transfusion Medicine*. Second ed. Philadelphia: Churchill Livingstone, 2007.
4. Bigbee WL, Vanderlaan M, Fong SS, Jensen RH. Monoclonal antibodies specific for the M- and N-forms of human glycophorin A. Mol Immunol 1983;**20**: 1353-62.
5. Bigbee WL, Langlois RG, Vanderlaan M, Jensen RH. Binding specificities of eight monoclonal antibodies to human glycophorin A--studies with McM, and MkEn(UK) variant human erythrocytes and M- and MNV-type chimpanzee erythrocytes. J Immunol 1984;**133**: 3149-55.
6. Blackall DP, Ugorski M, Pahlsson P, Shakin-Eshleman SH, Spitalnik SL. A molecular biologic approach to study the fine specificity of antibodies directed to the MN human blood group antigens. J Immunol 1994;**152**: 2241-7.
7. Risitano AM, Notaro R, Marando L, Serio B, Ranaldi D, Seneca E, Ricci P, Alfinito F, Camera A, Gianfaldoni G, Amendola A, Boschetti C, Di Bona E, Fratellanza G, Barbano F, Rodeghiero F, Zanella A, Iori AP, Selleri C, Luzzatto

L, Rotoli B. Complement fraction 3 binding on erythrocytes as additional mechanism of disease in paroxysmal nocturnal hemoglobinuria patients treated by eculizumab. Blood 2009;**113**: 4094-100.

8. Shoham-Kessary H, Naot Y, Gershon H. Immune complex-like moieties in immunoglobulin for intravenous use (i.v.Ig) bind complement and enhance phagocytosis of human erythrocytes. Clin Exp Immunol 1998;**113**: 77-84.
9. Powles T, Paterson A, McCloskey E, Schein P, Scheffler B, Tidy A, Ashley S, Smith I, Ottestad L, Kanis J. Reduction in bone relapse and improved survival with oral clodronate for adjuvant treatment of operable breast cancer [ISRCTN83688026]. Breast Cancer Res 2006;**8**: R13.
10. Moller G. Survival of Mouse Erythrocytes in Histoincompatible Recipients. Nature 1963;**199**: 573-5.
11. Mollison PL, Johnson CA, Prior DM. Dose-dependent destruction of A1 cells by anti-A1. Vox Sang 1978;**35**: 149-53.
12. Mollison PL. Blood-group antibodies and red-cell destruction. Br Med J 1959;**2**: 1123-30.
13. Moller G. Isoantibody-Induced Cellular Resistance to Immune Haemolysis in Vivo and in Vitro. Nature 1964;**202**: 357-9.
14. Klein H G ADJ, editor *Mollison's Blood Transfusion in Clinical Medicine* Victoria, Australia: Blackwell Publishing, 2005.
15. Brown DL, Lachmann PJ, Dacie JV. The in vivo behaviour of complement-coated red cells: studies in C6-deficient, C3-depleted and normal rabbits. Clin Exp Immunol 1970;**7**: 401-21.

16. Zimring JC, Cadwell CM, Chadwick TE, Spitalnik SL, Schirmer DA, Wu T, Parkos CA, Hillyer CD. Nonhemolytic antigen loss from red blood cells requires cooperative binding of multiple antibodies recognizing different epitopes. Blood 2007;**110**: 2201-8.

17. Auffray I, Marfatia S, de Jong K, Lee G, Huang CH, Paszty C, Tanner MJ, Mohandas N, Chasis JA. Glycophorin A dimerization and band 3 interaction during erythroid membrane biogenesis: in vivo studies in human glycophorin A transgenic mice. Blood 2001;**97**: 2872-8.

18. Anderson RA, Marchesi VT. Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide. Nature 1985;**318**: 295-8.

19. Anderson RA, editor *Red Blood Cell Membranes*: Dekker, 1989.

20. van Belzen N, Rijken PJ, Hage WJ, de Laat SW, Verkleij AJ, Boonstra J. Direct visualization and quantitative analysis of epidermal growth factor-induced receptor clustering. J Cell Physiol 1988;**134**: 413-20.

21. Ariotti N, Liang H, Xu Y, Zhang Y, Yonekubo Y, Inder K, Du G, Parton RG, Hancock JF, Plowman SJ. Epidermal growth factor receptor activation remodels the plasma membrane lipid environment to induce nanocluster formation. Mol Cell Biol 2010;**30**: 3795-804.

22. Yang S, Raymond-Stintz MA, Ying W, Zhang J, Lidke DS, Steinberg SL, Williams L, Oliver JM, Wilson BS. Mapping ErbB receptors on breast cancer cell membranes during signal transduction. J Cell Sci 2007;**120**: 2763-73.

23. Sanan DA, Anderson RG. Simultaneous visualization of LDL receptor distribution and clathrin lattices on membranes torn from the upper surface of cultured cells. J Histochem Cytochem 1991;**39**: 1017-24.

24. Chasis JA, Mohandas N, Shohet SB. Erythrocyte membrane rigidity induced by glycophorin A-ligand interaction. Evidence for a ligand-induced association between glycophorin A and skeletal proteins. J Clin Invest 1985;**75**: 1919-26.

25. Chasis JA, Mohandas N. Erythrocyte membrane deformability and stability: two distinct membrane properties that are independently regulated by skeletal protein associations. J Cell Biol 1986;**103**: 343-50.

26. Chasis JA, Reid ME, Jensen RH, Mohandas N. Signal transduction by glycophorin A: role of extracellular and cytoplasmic domains in a modulatable process. J Cell Biol 1988;**107**: 1351-7.

27. Chasis JA, Schrier SL. Membrane deformability and the capacity for shape change in the erythrocyte. Blood 1989;**74**: 2562-8.