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Investigations into the Development and Consequences of Red Blood Cell Alloimmunization

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Investigations into the Development and Consequences of Red Blood Cell Alloimmunization

By

Kathryn R. Pierce
B.S. New Mexico State University, 2006

Advisor: Dr. Mandy Ford, Ph.D.

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Investigations into the Development and Consequences of Red Blood Cell Alloimmunization

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Red blood cell transfusions can be a life saving therapy for patients with severe anemia, but they are not without risks. The generation of alloantibodies to RBC antigens can have serious adverse outcomes, including causing acute hemolytic transfusion reactions. In addition to transfusion, alloantibodies derived against RBCs during pregnancy can lead to hemolytic disease of the fetus and newborn, a condition with severe and sometimes fatal consequences for the developing fetus.

With the goal of better understanding red cell alloimmunization and incompatible RBC clearance, we generated a murine model with RBC-specific expression of the human KEL glycoprotein, which has been implicated in causing severe hemolytic transfusion reactions and HDFN. Transfusion of KEL RBCs into WT recipients consistently results in an anti-KEL antibody response, which is enhanced by recipient inflammation. In addition, we demonstrated that maternal exposure during pregnancy to fetal RBCs expressing paternally derived KEL antigens can similarly result in anti-KEL alloimmunization. Both transfusion and pregnancy-induced anti-KEL alloantibodies are boostable upon repeat exposure and are capable of binding to and causing antibody-mediated destruction of KEL RBCs. This destruction is accompanied by a systemic inflammatory response following subsequent transfusion or by decreased fetal survival in the case of pregnancy.

We further investigated the mechanisms of alloimmunization and found that removal of the spleen and more specifically, the marginal zone B-cells within the spleen, prevented alloimmunization to KEL RBCs in WT recipients. We also analyzed clearance patterns of KEL RBCs in immunized recipients and discovered a potential role for the complement protein C3 in clearance of incompatible RBCs as well as in antigen modulation, which may help to protect the remaining transfused RBCs from further immune mediated destruction.

These data have enhanced our understanding of the mechanisms of both the induction of anti-KEL alloantibodies as well as their clinical consequences in transfusion and pregnancy settings alike. This is the first murine model with the ability to recapitulate the essential features of a human response to a RBC antigen and will likely serve as a platform for the future development of targeted therapies to prevent or minimize the dangers of RBC alloimmunization.

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Introduction

HISTORY OF BLOOD IN MEDICINE

Blood in ancient culture and medicine

People love blood. This is evident in the modern day obsession with movies, television shows, video games, and art featuring violence, bloody crimes, and mythical creatures such as vampires; however, this fascination is not just a recent development. Several historical references indicate that although it is clear they have not always know its actual purpose, people have long had an obsession with blood. The involvement of blood in ceremonies and/or “medical” treatments has been described in the histories of many civilizations, including the ancient Egyptians, who were thought to have bathed in blood in efforts to restore their health and vigor, and in ancient Rome, where gladiators would drink the blood of other fallen gladiators in hopes of acquiring their strength [1, 2].

Historically, many have considered bloodletting as the preferred treatment for various ailments. One origination of the practice of bloodletting as a treatment for disease may have been through the teachings of the ancient philosopher and physician Hippocrates (460-370 B.C.E.), who described blood as being one of four humors that comprises the body, the other three being yellow bile, black bile, and phlegm. It was his theory that an imbalance of these humors, also known as temperaments, was the cause of human disease and in order to restore balance and become healed from a disease it was sometimes necessary to remove “excess” blood from the body[3].

Several other Greek physicians and/or philosophers that contributed to the early knowledge of blood and the circulatory system include Aristotle and Claudius Galenus. Aristotle suggested that the heart was the central organ of the body and the “seat of the soul”, and Galen was the first to suggest that arteries and veins are two distinct systems, and also noted that arteries carried “bright” blood and veins carried “dark” blood[4]. These theories indicate an evolving realization that human disease is a result a person’s nature and/or environment, and thus was a significant step in the study of physiology and medicine.

Because of the lack of scientific progress during the dark ages, the theories and teachings of Hippocrates and Galen regarding blood and its function persisted in Europe for approximately the next millennium. There are reports from within this time frame that claim that a pope, specifically Pope Innocent VIII, received a transfusion of blood from three young boys in 1492, but this is now considered to be unlikely. It had become fairly common practice in this time period for people to consume in attempts to “rejuvenate” or treat disease, so it is more likely that the blood was consumed by the pope, not transfused intravenously[5, 6].

In the 9th and 10th centuries C.E., Iranian physicians and scholars Abu Bakr Mohammad Zakariya Razi and Ali Ibn Abbas Majusi described several aspects of the circulation, as well as the structure and functions of the heart and the coronary arteries. In the 14th century, an Arabic physician named Ibn Nafis described the pulmonary circulation[4, 7, 8]. Credit of the discovery of pulmonary circulation, however, is often erroneously accredited to a 16th century Spanish

anatomist and theologist named Michael Servetus. In 1553 Servetus published a book, *Christianismi Restitutio*, which included a description of pulmonary circulation, and in which he also argued against the Galenic theories and denied the existence of the holy trinity. Servetus had previously translated many Arabic works, during which time he may have come across Ibn Nafis' manuscript on pulmonary circulation and included the descriptions in his own book[4, 9].

The function of the heart and its role in systemic circulation were described in a 1628 publication by the English physician William Harvey named *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus* (Anatomical Exercise on the Motion of the Heart and Blood in Animals), also known by its abbreviated title, *de Motu Cordis[10]*. Harvey is thought to have lectured on these theories several years earlier, but may have postponed publication due to the lingering belief in Galen's theories. Initially, many scientists and physicians rejected Harvey's work, but in time they gained more widespread acceptance, eventually leading a number of physicians to hypothesize on the possible benefits of transferring blood from one person to another to potentially prolong life or treat disease [11, 12].

The first blood transfusions

Andreas Libavious, a German doctor and chemist, became the first to suggest using blood transfusion as a therapeutic treatment for human disease in 1615. [13]. Later in the same century, Italian physicians Francesco Folli and

Giovanni Colle, as well as Francis Potter of England, similarly suggested that transfusion of blood may potentially prolong life [14-17].

The first to actually perform an intravenous transfusion of liquid into an animal is thought to be Sir Christopher Wren in 1657. Wren injected a variety of substances into the veins of dogs, including beer, wine, and opium, and observed the effects on their behavior and health, including observations of "suffering, vomiting, intoxication, and death". These experiments, while ethically questionable, demonstrated that substances that circulate in the bloodstream have systemic effects on an individual[18].

The first transfusions using animal blood were performed in the mid 1660's in England and France by Richard Lower and Jean Denis, respectively. Lower showed that after draining almost all of the blood from one dog, its life could be restored by a transfusion of blood from another dog. In these experiments, the blood was transferred by connecting the carotid arteries of one dog to another, nearly completely exsanguinated dog, which subsequently recovered. These experiments were significant in that they the suggested the potential use for transfusion of blood in a patient with severe blood loss[11, 19, 20].

Lower and Denis also performed the first animal to man transfusions in 1667, Lower using sheep's blood and Denis using lamb's blood. [21]. Denis explained his rationale for using animal blood instead of human blood because it was "less likely to be rendered impure by passion or vice". Their observations that the patients complained of symptoms including heat in the arm and kidney,

and black urine can now clearly be identified as symptoms of a transfusion reaction due to the cross-species incompatibility, but at the time the reason for these symptoms was unknown [22].

The most infamous transfusion experiment by Jean Denis nearly caused all transfusion research to be ended in Europe. In 1667, Denis transfused a man named Antoine Mauroy with blood from a calf in an attempt to cure him of mania, after which he complained of several symptoms indicating a transfusion reaction. A subsequent transfusion attempt was made two months later, and Mauroy died the next day. Denis was subsequently arrested and tried for his murder, however he was later exonerated when it was discovered that Mauroy's death was a result of poisoning by his wife. Despite this, the dangers of blood transfusion had been brought to public attention, and it was decided by the Faculty of Medicine in Paris, the French Parliament, the English Royal Society, and the Pope, that all transfusions were to be banned[15, 22].

The first human to human transfusions

Approximately 150 years after Denis' trial, an English physician and obstetrician named James Blundell proposed human to human blood transfusion. His initial interest and many of his theories were influenced greatly by another scientist, Dr. John Henry Leacock, who performed several experiments on dogs and cats and argued that it was important for blood donors and recipients to be of

the same species. Leacock also suggested transfusion could be used not only as a therapy for blood loss, but also for disease[23, 24].

Blundell's primary reasons for using transfusion as a therapy was to treat post-partum hemorrhage in women, but prior to attempting this he performed several experiments in animals to prove the usefulness and safety of the procedure. He made several important conclusions from his animal studies, including the danger of interspecies transfusion, the amount of air injected into the veins that could be tolerated, the temperature at which blood should be transfused, and the time after collection that it takes blood to coagulate. [25, 26].

Blundell's first human transfusion attempt took place in 1818 and involved several small transfusions of blood into a patient who was near death before the procedure. It was reported that the patient improved following the transfusion, but ultimately did not survive[27, 28]. After initially using syringes, Blundell noted that the blood would clot if left in the syringe for more than a few seconds. He sought to improve his technique by inventing a number of different devices to help facilitate the transfer of blood from the donor to the recipient[29].

Blundell attempted 10 more transfusions over the next decade, but only about half of his patients survived. This low success rate may have been influenced the initial poor prognosis of the patients selected prior to transfusion, or due to an inadequate volume of blood transferred. [29, 30]. Soon after, Blundell resigned his position from Guy's Hospital in London and retired in 1834 but held strong to the claim that some day people would realize that transfusion could have a therapeutic benefit. In 1949, an English physician named Walton

reviewed all of the cases of transfusion and concluded that the procedure was successful, with a mortality rate of 30%[25].

Later in the 19th century, Emil Ponfic and Leonard Landois characterized some of the dangers of xenotransfusion, however much of the transfusion research done for the rest of the 19th century was related to coagulation [2, 31, 32]. The process of blood defibrination, or removal of the clot after it forms, prior to transfusion was described by Sir Thomas Smith in 1873 and was sometimes used. In addition, a variety of substances, including sodium bicarbonate, sodium phosphate, and oxalic acid were tested as anticoagulants, but were found to be quite toxic in humans. In 1894, Sir Almroth Wright, discovered a solution of citrate salts that prevented coagulation “indefinitely”, however it took over 20 years before the use of citrate as an anticoagulant was used in any type of transfusion procedure[33, 34].

Transfusion in the Early 20th Century and the Discovery of Blood Groups

Although transfusion in humans had been deemed an acceptable treatment in the mid 19th century, and the development of anticoagulants and use of sterilization techniques made the procedure safer and easier to perform, transfusions were still rare due to the dangerous and potentially fatal reactions often experienced by patients following the procedure. It was not until the work of Karl Landsteiner (1868-1943 C.E.), an Austrian physician and scientist, that the cause of these transfusion reactions became clearer. Landsteiner observed

that serum from one donor could agglutinate red cells from another donor *in vitro*, and in 1900, he published a manuscript describing 3 blood groups, A, B, and O. Soon after, Alfred vonDeCastello and Adriano Sturli discovered the fourth blood group, AB[35]. At approximately the same time, a Czech serologist named Jan Jansky also discovered the same four blood groups, but named them I, II, III, and IV[36].

It was still a number of years before cross matching for blood group prior to transfusion became standard practice. Ruben Ottenberg, a physician at Mt. Sinai in New York, was the first to routinely perform cross matching prior to transfusion, and observed a much lower rate of transfusion reactions in his patients. Ottenberg also proposed the idea of universal donors and recipients in 1911[37].

In 1914, nearly 20 years after Sir Amroth Wright discovered that citrate salts could keep blood from clotting, Albert Huston from Brussles, Luis Agote from Argentina, and Richard Lewishon from England all discovered that sodium citrate could be used as an effective and safe anticoagulant[38-40]. Over the next several decades, significant progress was made in the optimization of anticoagulant solutions and the preservation of red blood cells, including the addition of adenine and glucose to the preservative solution as well as determining the proper pH and temperature for optimal blood storage. With these improvements, blood went from being able to be stored for only a few days to a several weeks (up to 42 days)[39]. This became especially important during the wars, as blood was often needed to transfuse wounded soldiers but donors

were not as readily available. The first person to establish a repository of collected blood for this purpose was Oswald Robertson in 1918[41]. There were no official American “blood banks” until Bernard Fantus established one in Cook County Illinois in 1934[42].

Other important advancements that occurred in the mid-20th century include the discovery of additional blood group systems and further improvements in blood collection, storage, and processing. A report of hemolytic disease of the fetus and newborn (HDFN) by Phillip Levine an R.H. Stetson led to the characterization of the Rh blood group system by Karl Landsteiner and Alexander in 1940 [43, 44]. Further research on blood group antigens was performed by Ruth Sanger and Robert Race, who published one of the most important books in transfusion medicine, *Blood Groups in Man*, in 1950 [45]. Additional developments include the invention of the plastic bag used for blood collection and storage by Carl Walter in 1950, the creation of the antiglobulin (Coomb’s) test by Robin Coombs, and the development of techniques by Edwin Cohen that made it possible to separate blood components. Additionally, leukoreduction filters were developed in 1962 and decreased the rate of febrile non-hemolytic transfusion reactions as well as the risk of some transmitted infections[2, 46].

Transfusion medicine in the last 50 years

The field of transfusion medicine abruptly changed in the early 1980's after the emergence of the HIV virus and the discovery that it could be transmitted through blood. The prevention of HIV transmission, as well as that of other blood borne diseases such as hepatitis C and malaria, became the primary focus of transfusion research during the last part of the 20th century. With the introduction of more thorough donor screening as well as improved testing measures for infectious agents in donated blood, transfusion medicine research in recent years has turned its focus once again to non-infectious serious hazards of transfusion.

RED BLOOD CELL BIOLOGY AND MODERN TRANSFUSION MEDICINE

Red blood cell structure and function

Red blood cells (RBCs), or erythrocytes, have the primary function of delivering oxygen from the lungs to the tissues and carbon dioxide from the tissues back to the lungs. Erythrocytes are a unique cell type, as they are little more than just a plasma membrane surrounding a large amount of hemoglobin, which is the protein primarily responsible for binding to the oxygen molecules during transport. In their mature form, red blood cells have no nucleus and do not have the ability to synthesize new proteins, thus all of the proteins that a red

blood cell will ever express have been generated from the start of erythropoiesis through the reticulocyte (pre-erythrocyte) stage[47].

Another unique characteristic of red blood cells is that their plasma membrane and internal cytoskeleton must be very strong, but also allow a for a high amount of deformability so that they are able to effectively travel through the narrow spaces of the microvasculature in order to deliver oxygen to all tissues[48]. Similar to other cell types, the plasma membrane of erythrocytes is composed of lipids, proteins and carbohydrates, but the interactions between the membrane and the internal cytoskeleton gives the erythrocyte the strength and deformability it requires. The internal cytoskeleton is primarily made up of a fibrous protein called spectrin, which forms a lattice type structure and attaches to the membrane proteins band 3 and Glycophorin C via another protein called ankyrin, as well as through associations with a complex of proteins that includes actin and tropomyosin. Mutations in either spectrin or ankyrin of can cause defects in red cell shape and deformability, leading to a variety of genetic diseases[49]

Blood group antigens

Red blood cells cannot synthesize new proteins once they are mature erythrocytes, but that does not mean that they do not express any surface proteins. In fact, red cells have many different types of glycoproteins, glycolipids, and carbohydrates on their cell surface. In the most recent analysis by the

International Society of Blood Transfusion, 328 blood group antigens exist, 284 of which are categorized into 30 different blood group systems[50]. The functions of the red cell surface molecules that contain these antigens vary widely. Some act as channels or transporters for water or ions, some play a role in adhesion or structural integrity, some have enzymatic activity, and some function as receptors[51].

The immunogenicity of blood group antigens depends on several factors, the most general of which is the type of molecule containing the antigen. Proteins are typically more immunogenic than carbohydrates, which in turn are more immunogenic than molecules such as lipids and nucleic acids. Protein antigens generally induce a T-dependent immune response, requiring the involvement of both B-cells as well as $CD4^+$ T-cells, and result in the production of first IgM, then IgG antibodies. Conversely, carbohydrate antigens often contain repeating epitopes and thus elicit a T-independent B-cell response, resulting in the production of IgM antibodies with less class switching to IgG[52].

Most blood group antigens become immunogenic as a result of mutations or polymorphisms in various red cell surface molecules. The difference between two individuals may be as small as one amino acid change, but can result in alterations in the sequence of linear polysaccharides or polypeptides, or may cause conformational changes in proteins due to alterations in folding. Any of these changes may result in the exposure or generation of a B or T-cell binding site, which can induce an immune response in a recipient lacking that particular polymorphism[48].

The most clinically significant blood group system known is ABO, which as previously mentioned was also the first to be discovered by Landsteiner in the early 20th century. Unlike most blood group systems, in which antigens differ by slight amino acid changes from person to person, in the ABO system the entire A or B protein is either present or absent on an individuals red cells. Individuals are classified into "blood types" by whether they express one, both, or neither of the A or B antigens. Those lacking expression of either of these particular carbohydrate antigens make natural IgM alloantibodies against the other, resulting in agglutination of red cells expressing that antigen upon exposure[52].

The second most clinically significant blood group system is Rh, or Rhesus. This system includes approximately 50 antigens, the most immunogenic of which is RhD. The approximately 50 other Rh antigens besides D are much less immunogenic, however there are some that are still considered clinically significant, including the antithetical antigens C/c, and E/e. Similar to the ABO system, people are characterized by the presence or absence of the entire RhD molecule on their red cells surface, denoted by a + or – following their ABO classification. Different from the ABO system, however, is the way by which alloantibodies are generated to the RhD antigen. Immunization can only occur upon exposure of an RhD- individual to RhD+ red cells, and the primary immunoglobulin isotype generated in response to the RhD antigen is IgG[48, 53]. This presents a risk not only in a transfusion setting, but also in pregnancy. Since IgG antibodies can cross the placenta, hemolytic disease of the fetus and newborn (HDFN) may occur when an RhD^- woman is pregnant with an RhD^+

fetus and makes anti-RhD antibodies, which can cross the placenta and attack the fetal RBCs. Prior to the introduction of RhoGAM, an intravenous Rh immunoglobulin treatment given to RhD^- pregnant women with the potential to have an RhD^+ fetus, the incidence of anti-RhD antibody production in the mother was quite significant, occurring in approximately 15-20% of RhD^- mothers. Since its successful clinical trials were reported and the drug RhoGAM was licensed for use in 1968, it is estimated that approximately 88-95% cases of RhD immunization during pregnancy have been prevented[54, 55]

As previously mentioned, many blood group antigens other than those in the ABO and Rh systems exist that have the ability to induce alloantibodies. Some, such as those against Kell, Duffy, and Kidd, are very clinically significant and can cause mild to severe transfusion reactions and/or hemolytic disease of the fetus and newborn. Others, such as those against Chido-Rogers, and Knops only cause a mild reaction, if any, and are considered not to be clinically significant. Many fall somewhere in between, and typically only become a clinical issue in multiply transfused patients that have also made an immune response to many other blood group antigens as well. [52, 56]

The Kell Blood Group System

Besides ABO and RhD, Kell is the most immunogenic blood group system. Antibodies against Kell were first discovered in 1946 due to a case of hemolytic disease of the fetus and newborn, and Philip Levine later described the

Kell antigen in an article in Science in 1949.[57, 58] In addition to hemolytic disease of the fetus and newborn, antibodies against Kell can cause severe hemolytic transfusion reactions. Clinically, antibodies against Kell are of the highest concern when transfusing a patient, since blood is always matched for ABO and RhD but typically not for Kell. The major antigens in the Kell system are Kell and Cellano, which differ by one amino acid, but the system also contains at least 25 other antigens, making Kell one of the most polymorphic blood group systems.[59]

The Kell protein is a Type II, single pass membrane glycoprotein. It includes a 665 amino acid extracellular portion linked to a 47 amino acid cytoplasmic portion by a 20 amino acid long cytoplasmic domain. Functionally, Kell is a zinc endopeptidase, which can cleave peptides such as endothelins to activate them. Endothelin 3 is the primary substrate for Kell, and plays a role in vasoconstriction and nervous system development when activated. Kell is likely a redundant enzyme, as lack of Kell causes no decrease in activated plasma endothelins and there is no apparent defect in the patients.[60, 61]

Kell is very polymorphic and mutations at several sites within the gene can cause single amino acid substitutions resulting in a number of antithetical antigens, which can differ between individuals. For example, the major antigens in this system, Kell(K) and Cellano(k), differ by one amino acid at position 193, either containing a methionine or threonine. The majority of the population expresses the Cellano form, depending on ethnic background the Kell form only occurs in approximately 2-9% of the population. Other “high incidence”

antithetical antigens in the Kell system include $Kp^a/Kp^b/Kp$ and Js^a/Js^b. A total absence of Kell antigens also sometimes occurs; this phenotype is known as K_0. Furthermore, Kell is typically expressed in the membrane linked with another protein, XK, via a disulfide bond, and lack of XK results in very low expression of Kell in the membrane. This condition is known as McLeod syndrome and is characterized by several muscular and neurologic defects.[62]

Benefits and risks of blood transfusion

Transfusion of blood or blood products can be a life saving therapy for people who suffer from anemia, thrombocytopenia, or coagulopathy either due to hematological disorders or due to blood loss from trauma or surgery. Acute blood loss not only results in the decreased ability of the body to transport oxygen to organs and tissues, but the reduced blood volume also may cause other symptoms such as a dangerous drop in blood pressure, increase in heart rate, respiratory distress, dizziness, and shock. Diseases that result in the decrease or lack of functional erythrocytes, platelets, or other blood cells similarly interfere with oxygen delivery, clotting, and other necessary functions. In these situations, transfusions of red cells, platelets, plasma, or other blood products can help replace lost blood volume, restore normal oxygen delivery, and reverse the symptoms of anemia[48, 63].

Despite its advantages, there are also many risks associated with blood transfusion. One of the greatest and most obvious risks is the potential transfer

of an infectious agent from donor to recipient through the donated blood. Historically, the transfusion transmitted infectious diseases (TTID) that were the most common were syphilis and hepatitis, caused by either the hepatitis A, B, or C virus[64]. Arguably the worst and most infamous TTID, however, was HIV. When the first cases of AIDS were documented in the early 1980s, it was thought that the disease was only transmitted between homosexual men[65, 66]. It was soon discovered that the disease could also be transmitted through blood transfusions and “gay related immune deficiency (GRID)” was renamed “acquired immune deficiency syndrome (AIDS)”[67] Although it was recognized that this disease was likely being transmitted via transfused blood, the causative agent of the disease was still unknown at this time. Major changes in donor selection were instituted in efforts to avoid collecting blood from “high risk” individuals [68]. By 1984, the virus responsible for AIDS was discovered, and the development of an antibody test to identify carriers of the virus soon followed[69].

Many additional pathogens have the potential to be transmitted through transfused blood. Bacterial contamination is a continuing concern, and can occur not only due to an infection within the donor’s blood, but also due to errors in sterile collection techniques[70]. Parasites such as *Plasmodium* (malaria), *Trypanosoma* (Chagas disease), and *Babesia* (babesiosis) can also be transmitted through transfusion, but such events are rare in non-endemic countries. Unlike the strict testing required for the detection of other pathogens, transmission of parasitic infections through blood is avoided primarily by prohibiting individuals who visited endemic regions from donating blood for a

period of time[64]. Testing measures for emerging disease threats are continuously added, for example screening measures have recently been put into place for pathogens such as West Nile Virus and Creutzfeldt Jacob disease. Overall, between the strict donor criteria, improved collection methods, and thorough pathogen screening, the risk of an infectious disease being transmitted through transfusion has greatly decreased[71].

Unfortunately, transmission of infectious disease not the only risk associated with transfusion. All other risks are called noninfectious serious hazards of transfusion, or NISHOT. Some of these risks may be due to human error, for example mistransfusion of the wrong blood type, or transfusion of an inappropriate amount of blood, known as over or under transfusion. Others are typically classified either as immune-mediated or non-immune mediated. Some of the non-immune risks include iron overload, transfusion associated circulatory overload (TACO), reactions to the red cell storage lesion, metabolic complications, and septic transfusion reactions. Immune-mediated risks vary widely, ranging from fever or anaphylaxis to transfusion-associated graft vs host disease (TA-GVHD), transfusion related immunomodulation (TRIM), and alloimmunization against antigens that differ between the donor and recipient, which can lead to hemolytic transfusion reactions[72].

There is a growing concern that the over-utilization of transfusion as a treatment for anemia or blood loss during surgery is actually causing more detrimental consequences to the patient than previously recognized. Several studies have reported that transfusion is associated with increased morbidity and

mortality, higher rates of infections and sepsis, longer hospital stays, and worse outcomes in surgery patients[73-75]. Furthermore, studies comparing groups that restricted the use of transfusions vs groups that did not restrict transfusions showed that lower rates of mortality and post-surgery complications occurred in the group that restricted transfusions[76]. These and other related studies indicate that although transfusion is a common procedure, there is much that is still unknown about its risks and further research will be required to better understand the consequences of transfusing red blood cells or other blood products[77]

THE IMMUNOLOGY OF TRANSFUSION MEDICINE

Alloimmunization to Red Cell Antigens

Red blood cells are a unique type of antigen. Unlike antigens found on other immunogens such as microorganisms or transplanted organs or tissues that drain into lymph nodes, red cells are restricted to the circulation, and their antigens are generally only seen by immune cells within the spleen and liver. Also, compared to most other antigens, which induce an immune response to nearly every exposure, red cells antigens are typically fairly weak immunogens with immunization occurring only in about 1-3% of transfusions in non-chronically transfused patients and 3-10% in those receiving repeated transfusions[78].

This excludes ABO and RhD, as these antigens are matched to the recipient prior to every transfusion. Individuals lacking the A or B molecules on their red cells have natural IgM antibodies against them, resulting in rejection of a mismatched transfusion in nearly every case. Rates of immunization to RhD are also much higher than other red cells antigens, occurring in approximately 30-80% of D exposures in D negative recipients, since the difference between donor and recipient is not simply an amino acid change but the presence or absence of the entire RhD molecule[79].

Whether alloimmunization to a red cell antigen occurs depends on several factors, including specific characteristics of the antigen such as the location of the epitope relative to the membrane or other antibody binding sites, the chemical or physical changes introduced into the protein by the particular amino acid substitution, and the degree of antigenic difference between the donor and recipient [80]. Specific characteristics of the host are also involved in alloimmunization, such as whether the recipient expresses HLA molecules capable of presenting a peptide containing the particular epitope. If the particular peptide does not fit into the binding cleft of the HLA molecule, the antigen presenting cells will be unable to present the peptide to T cells, thus preventing the development of an antibody response [81, 82]. Blood group antigens that have been discovered to have a specific HLA restriction include Fy^a (Duffy A) and Jk^a (an antigen in the Kell blood group system)[83, 84]. Another host factor that has been shown to affect alloimmunization rates is the disease or inflammatory status of the recipient. In general, patients who require a

transfusion generally are quite sick, as is the case with most who receive chronic transfusions, or have a higher than normal level of systemic or local inflammation, as is the case with trauma or surgery patients. Sometimes, however, due to certain types of diseases or medications, patients may be immunosuppressed when receiving a transfusion. These varying levels of host inflammation have been shown to have a significant effect on alloimmunization to transfused red cells[85-88].

Rates of alloimmunization and other complications from transfusion can also be caused by factors independent of either the donor or recipient; one such example is red cell storage[89]. The FDA guidelines state that stored RBCs must have a 24-hour post-transfusion recovery rate of 75%, and determined that such RBC survival rates could be achieved with units that had been stored for up to 42 days in the currently approved anticoagulant storage solutions. It has become clear, however, that post-transfusion survival is not necessarily indicative of the quality of the red cells being transfused, and that older blood may cause many negative effects when given to a patient[90, 91]. Many physical, biochemical, and immunological changes occur to a red cell in storage, which may contribute to such negative effects. These effects, termed the “RBC storage lesion”, include changes in red cell shape and membrane deformability due to the formation of vesicles, decrease in ATP and 2,3-DPG which affects the oxygen carrying capacity of the red cell, a drop in nitric oxide (NO) which also may affect oxygen delivery, and changes in cation pump activity. Some of these effects are reversible to different extents once the red cells reenter circulation, but other

changes that occur in storage such as aggregation or fragmentation of proteins and lipids in the membrane due to oxidative damage[92-94]. Many clinical studies have indicated that transfusion of red cells that have been stored for longer periods of time is associated with worse patient outcome, increased rates of sepsis, more frequent complications after surgery, and higher morbidity and mortality [95-97]. Conversely, other clinical studies have shown that there are no negative effects associated with transfusion of older vs fresher blood [98, 99]. Mouse models of red cell storage have also shown higher rates of alloimmunization and other complications following transfusion of stored blood[100-102]. These findings indicate that the FDA guidelines for blood storage are outdated and should be modified to reflect the more current understanding of red cell storage and its associated clinical consequences.

Another aspect of red cell alloimmunization that remains incompletely understood is the phenomenon of “responders” and “non-responders”. It is widely thought in the field of blood banking and transfusion medicine that a population of patients exists who becomes alloimmunized to red cell antigens at a much higher rate than the average patient population, and continues to have higher rates of immunization to subsequent transfusions as well [85, 103]. For example, it has been observed that sickle cell patients have an abnormally high rate of alloimmunization, even after taking into consideration that they receive chronic transfusions and thus are frequently exposed to new red cell antigens[104, 105]. A recent retrospective study analyzing blood bank data found strong evidence that there exists a population of previously alloimmunized

patients with significantly higher risk of subsequent alloimmunization compared to other transfused patients, and also suggested that this did not depend on the number of previously existing antibodies[106]. Extended phenotype matching of donor and recipient red cells is performed for “responders”, and is currently the only preventative measure to avoid the development of additional alloantibodies in these patients. It may help lower the risk of alloimmunization, however it is not foolproof. The specific cause of why responders and non-responders exist has still not been elucidated, and therefore the development of a prophylactic treatment is still not possible.

The role of the spleen in alloimmunization

As previously mentioned, red cells are unique in that they are normally isolated within the vasculature. Because they are contained within this compartment, the only peripheral lymphoid organ that antigens within blood come into contact with is the spleen. Red cells also pass through the liver, which contains many immune cells, however due to the constant exposure of these immune cells to intestinal or food-derived antigens, the liver environment is generally more tolerogenic than immunogenic[107].

In contrast to the liver, the spleen is crucial for the generation of immune responses to antigens within the blood, including not only red cell antigens but those associated with blood borne pathogens as well. The structure of the spleen makes it ideally suited to filter the blood to remove old or damaged

erythrocytes, and to detect any foreign antigens. Blood enters the spleen via the splenic artery into an area known as the red pulp. This region of the spleen is macrophage rich and has a structure designed to remove old or damaged red cells. The red pulp macrophages not only phagocytose the old erythrocytes and remove them from circulation, but also recycle the iron from within[108].

The surviving cells then cross the marginal zone by an active process involving G-coupled protein receptors[109]. Several unique cell types reside in the marginal zone, including marginal zone b-cells, marginal zone-macrophages, and marginal zone metallophilic macrophages, along with other standard immune cell types such as dendritic cells and lymphocytes. These cells express standard pattern recognition receptors as well as others such as specific scavenger receptors, which allow them to recognize antigens that are more likely to be in the blood. Marginal zone b-cells are phenotypically and functionally distinct from follicular B-cells, and have been shown to become rapidly activated following exposure to a blood borne antigen and produce IgM [110]. A role for marginal zone b-cells in the activation of CD4+ T-cells has also been described. [111]. Furthermore, there is evidence that marginal zone b-cells themselves can undergo class switching and thus may be able to produce not just IgM, but IgG as well[112, 113].

The white pulp of the spleen is similar to a lymph node in that it has both T-cell zones, called the periarteriolar lymphoid sheath in the spleen, and B-cell zones where activated B-cells undergo class switching and somatic hypermutation within follicles. Unlike lymph nodes, there are no efferent

lymphatic vessels by which lymphocytes can leave the spleen, so lymphocytes either remain in the white pulp or migrate through the marginal zone to the red pulp of the spleen, where it is thought that they may be able to reenter the bloodstream through the venous sinuses in the red pulp along with the red cells. [114-116].

Although it is clear that the spleen is involved, the specific role of the spleen and the particular cell types required for alloimmunization to red cell antigens have not been entirely elucidated. Some recent studies have suggested that marginal zone b-cells are critical in the response to blood borne antigens[117, 118]. Some patients with autoimmune hematological disorders undergo surgical splenectomy in order to avoid further autoantibody formation, which demonstrates the understanding that the spleen is involved in alloimmunization [119]. The use of a transgenic mouse expressing a model antigen have also shown that primary alloimmunization to red cell antigens does not occur in splenectomized recipients[120]. Furthermore, additional recent studies have demonstrated that the immune response to certain blood borne pathogens can be prevented by specifically depleting marginal zone B-cells[121-123]. These data suggest a potential role for marginal zone b-cells in the immunization not only to blood borne pathogens, but to red cell antigens as well.

Consequences of red cell alloimmunization

Once an individual has become immunized against a red cell antigen, a subsequent transfusion of blood with the same antigen often results in immune-mediated destruction of the red cells, however this is not the inevitable outcome. In some cases, the patient experiences no clinical symptoms upon transfusion of the incompatible unit, and the red cells continue to circulate normally, despite being bound by antibody. These antibodies are termed “clinically insignificant”, and appear to be associated with particular blood group antigens, such as Chido-Rogers, Knops, and JMH[124].

Antibodies that do cause destruction of red cells are considered “clinically significant”, and can cause a hemolytic transfusion reaction upon a subsequent transfusion with blood containing the same antigen. An acute hemolytic transfusion reaction (AHTR) is typically characterized by rapid intravascular hemolysis immediately after or within a few hours following transfusion. Other inflammatory mediators such as IL-1, IL-6, IL-8, TNF-α, and histamine are produced as well, contributing to a number of severe symptoms observed in AHTR which include vasodilation, hypotention, fever, chills, pain in the chest, abdomen, back or flank, nausea, hemoglobinuria, acute renal failure, disseminated intravascular coagulation, shock, and sometimes death [52, 125].

Symptoms of a delayed hemolytic transfusion reactions (DHTR) typically do not appear until 3-10 days following a transfusion. In DHTR cases, the patient typically has made a response to an RBC antigen after a previous exposure, but the antibody levels in their serum decreased to the point of non-reactivity with the donor unit prior to transfusion. Transfusion of the unit triggers an anamnestic

response, and the secondary immune response rapidly produces higher levels of antibodies within a matter of days, which can lead to a combination of intravascular and extravascular hemolysis of the transfused red cells. Symptoms experienced during a DHTR are generally not as severe as an AHTR, but in some cases can still be quite dangerous[126].

For most patients, hemolytic transfusion reactions or other complications associated with alloimmunization to red cell antigens are quite rare. However, for the patients with diseases or hematological disorders that requires them to be frequently transfused, these events can be a major concern. Since there is currently no therapeutic intervention available to prevent the generation of immune responses to red cell antigens, these patients continue to generate alloantibodies against multiple antigens, making it increasingly more difficult, or in some cases impossible, to find a compatible unit. Phenotypic matching between donor and recipient red cells is performed in some cases, which may help to avoid further alloimmunization, but again it is a game of evasion instead of prevention. A more thorough understanding of the mechanisms of the immune response against red cell antigens may lead to the eventual elucidation of a therapeutic target or option that could prevent the alloimmune response to transfused red cells or other blood products.

Hemolytic disease of the fetus and newborn

Alloantibodies against red cell antigens can cause problems not only in transfusion but also in some cases in pregnancy as well. Hemolytic disease of the fetus and newborn (HDFN) is a condition in which a pregnant mother makes alloantibodies against red cell antigens of the fetus, which transfer through the placenta and destroy the fetal red cells. This has severe consequences for the fetus, which can lead to major physical and developmental defects and in some cases, death. Historically most cases of HDFN were due to RhD incompatibility between mother and fetus, but rates have decreased greatly due to the implementation of RhoGAM (anti-RhD immunoglobulin) for use in pregnant mothers at risk for RhD immunization. Besides RhoGAM, however, there is no prophylactic treatment for HDFN so alloimmunization against other red cell antigens cannot be prevented and antibodies generated against these fetal or transfused red cell antigens still may pose a risk for the developing fetus[127].

With the prevalence of anti-RhD alloantibodies greatly reduced due to RhoGAM, anti-Kell has emerged as one of the alloantibodies at high risk for causing severe HDFN. Kell alloimmunization only occurs in roughly 0.15% of pregnancies, however approximately 10% of severe fetal anemias are attributed to anti-K. The anemia caused by anti-K alloantibodies is more often due to suppression of erythropoiesis than hemolysis in the fetus, a condition for which the only feasible treatment option available is an intrauterine transfusion, which is an invasive and potentially hazardous procedure[128, 129].

Immune destruction of red blood cells

When an immunized individual receives a transfusion of incompatible blood, immune destruction of the transfused red blood cells is generally thought to occur by two main mechanisms. The binding of the antibodies to the antigen does not in itself cause damage, but they can facilitate destruction of the red cells either by binding to Fc receptors on phagocytes, which induces extravascular destruction in the spleen and/or liver, or by triggering the complement cascade, which can lead to intravascular rupture of the erythrocytes within the bloodstream [130].

Extravascular destruction is the primary route of red cell clearance in delayed hemolytic transfusion reactions, but can also occur during an acute reaction as well. This type of destruction is mediated by the attachment of antibody coated RBCs to Fc receptors on phagocytes in the spleen or liver. Antibodies are typically of the IgG isotype, and whether hemolysis occurs also depends on IgG subtype, with IgG1 being most likely to cause red cell destruction, followed by IgG3 and in rare cases, IgG2 [131]. IgG4 subtype antibodies that can bind to RBCs have been reported, but are not known to cause hemolysis and thus are not usually considered clinically significant [132].

Macrophages in the spleen and liver express all Fcγ receptors, FcγRI, FcγRII, and FcγRIII,which are all capable of binding IgG1 as well as other IgG subtypes with varying affinities. The highest affinity receptor for most IgG subtypes, including IgG1 is FcγRI, followed by the lower affinity receptors FcγRIIa

and FcγRIII. FcγRIIb has an inhibitory instead of an activating cytoplasmic domain; therefore it is not involved in Fc-mediated destruction of red cells, but plays an important role in inhibition of B cell responses [133, 134].

The two mechanisms by which macrophages can destroy an Fc receptor-bound antibody coated red cell are through engulfing the cell or by releasing cytolytic enzymes that lyse the bound red cell. The specific factors that determine whether phagocytosis or lysis will occur are not completely understood, but they are thought to include the quantity of antibody bound to the red cell and whether there are C3 molecules bound to the surface of the red cells that can be recognized and bound by complement receptors present on the macrophages. Some data suggest a correlation between high levels of bound antibody and cell lysis, but other conflicting data dispute this correlation[135, 136]. Studies also have shown that the presence of bound complement, along with bound antibody may provide synergistic signals through both Fcγ and complement receptors that trigger phagocytosis [137, 138]. In addition, extravascular destruction may be affected by the state of the RES itself. Macrophage function can vary considerably depending on their environment, for example the presence or absence of cytokines or other inflammatory mediators, so it is reasonable to expect that certain diseases states or the general health of the patient may affect the activity or effectiveness of macrophages within the RES[139, 140]. Studies have even shown that while macrophages are the primary cell type responsible for red cell consumption under normal physiological conditions, in the presence of certain types of inflammation, consumption of red

cells by splenic dendritic cells significantly increases, which can have downstream effects on alloimmunization[85].

In contrast to extravascular hemolysis, intravascular hemolysis typically occurs during an acute hemolytic transfusion reaction and is primarily mediated by complement. The complement system plays an important role not just in causing intravascular hemolysis of the transfused red cells by triggering the complement cascade to the formation of the membrane attack complex, but also in other post transfusion events by releasing inflammatory mediators which contribute to processes such as anaphylaxis, vascular permeability, leukocyte chemotaxis, and platelet activation [141, 142].

Either IgM or IgG antibodies may trigger complement, however IgM is typically far more efficient due to its superior ability to bind C1q, the initial antibody binding subunit. This in part is because C1q requires two IgG molecules in close proximity to each other (approximately 20-30nm) in order to bind, however only one molecule of IgM is required. Once bound to antigen, IgM changes its conformation in a way that allows both binding sites of C1q to bind to a single molecule [143]. Alloantibodies that bind complement are often naturally occurring IgM antibodies, such as anti-A or anti-B, but some IgG antibodies bind complement as well, including Duffy a (Fy^a) and the Kell system antigens K, Jk^a, and Jk^b [144, 145].

C3 ligation can cause destruction of red blood cells not only through intravascular lysis, but can also play a role in the sequestration of the red cells within the reticuloendothelial system. Spleen and liver macrophages express

complement receptors CR1, CR3, CR4, and CRIg, which recognize several forms of complement subunits including C3b and iC3b. As with Fc mediated red cell destruction, phagocytosis may not be triggered solely due to C3 coated red cells binding to the macrophages through complement receptors, but may require the synergistic effect of antibody bound to Fc receptors as well. Regardless of whether they are destroyed or just sequestered within the spleen and/or liver, however, the red cells are effectively removed from circulation [140, 146].

Additional mechanisms of red cell destruction that do not involve either Fc receptors or the involvement of complement have also been proposed. For example, it has been demonstrated that antibody binding in itself may destabilize the membrane, potentially due to the induction of Ca^{2+} influx, causing eryptosis, or programmed cell death, of the red cell. Antibody binding has also been shown to induce phosphatidyl serine expression on the red cell membrane, which can trigger their destruction by scavenger receptor expressing phagocytes. [147, 148]. Furthermore, a recent study also reported that agglutination of antibody bound red cells may lead to their sequestration and subsequent destruction by phagocytes within the RES[149].

Escape from immune-mediated clearance

As previously mentioned, destruction is not the inevitable outcome of antibody binding to transfused red cells. Some red cells bound by non-clinically significant alloantibodies can continue to circulate normally despite being bound

by antibody. In some cases, however, red cells that have been bound by alloantibodies that typically would cause destruction of the red cells continue to remain in circulation by undergoing antigen modulation[150-152]. Also called “antigen loss”, “antigen suppression”, “antigen depression”, or “weakened antigenicity”, antigen modulation is a process by which cells alter the target antigen in order to evade detection by the immune system. This phenomenon has been shown to occur in multiple cell types such as red cells, platelets, and transplanted tissues and organs, as well as in diseases such as Myasthenia Gravis and Pemphis Vulgaris[124]. In red cells, antigen modulation has been observed with a number of different antigens, but appears to most frequently occur in the Kell system [153, 154].

Over 40 years worth of case reports describe antigen modulation in red cells, yet the mechanisms by which these phenomena occur are still not fully understood. A variety of explanations have been proposed, including antibody-induced modification of erythropoiesis to produce only antigen negative erythrocytes. This mechanism may occur in some situations, such as autoimmune hemolytic anemias, however it does not account for the incompatible transfusion setting, where the target red cells come from a separate individual and are not generated within the recipient[155]. Another hypothesis is the physical removal of all or part of the molecule containing the antigen from the red cell membrane, which has been demonstrated to occur in the Kell system and has also been reported in the Lutheran and Gerbich systems [156].

Another possibility is that the molecule or epitope is not altered, but is somehow masked or hidden from the immune system, which has been shown to occur with some complement binding alloantibodies, including Kell. When bound to a cell, C3b undergoes a series of cleavage reactions, first forming iC3b which can no longer activate additional C3 molecules but can still be bound by complement receptors on phagocytes. Additional cleavage reactions form C3dg and C3d, which are also inactive but now can no longer be recognized by complement receptors on phagocytes. C3dg and C3d remain covalently bound to the membrane and may mask the target antigen, potentially protecting it from immune mediated destruction[157-160]. Additionally, C3 mediated destruction may also be avoid by the inhibition of complement via complement regulatory proteins[161, 162]. Either mechanism may alter the antigen or antibody in such a way that immune mediated destruction is prevented. Recent studies using animal models have begun to elucidate potential mechanisms of antigen modulation, however the specific mechanisms of antigen modulation associated with particular blood group antigens has yet to be determined [163, 164].

ANIMAL MODELS OF RED CELL ALLOIMMUNIZATION

Though it would be ideal to study human disease in humans, due to ethical limitations this is not feasible. Therefore, animal models have long been used for the study of human disease, and transfusion medicine is no exception.

Initial research on the immune response to blood transfusion was performed using a number of different animals, including dogs, cats, rabbits, monkeys, sheep, rats, and mice[165-167]. Some early red cell immunization studies looked at the effect of transfusing human or sheep RBCs into mice, however this system is complicated by several factors, including that the human or sheep RBCs are much larger than mouse RBCs and also that they are xenoantigens in the mice, which may alter the immune response[168]. Allogeneic blood transfusion between mice emerged as the most useful model to study red cell antigens due to the generation of many inbred, knockout, and transgenically modified strains, their relatively low cost and ease of use, and because there are also a wide array of immunological tools and reagents available to study the immune responses within them[169].

Several mouse models specific for the study of alloimmunization to and clearance of transfused red cells. Some of these transgenic mice express human blood group antigens such as Duffy^{b} and hGPA, and some express model antigens such as HEL (hen egg lysozyme) or HOD, which is a fusion protein containing the sequences for hen egg lysozyme, ovalbumin, and Duffy^{b} [170]. These models have been useful in studying alloimmunization to red cell antigens, however each has aspects that make them differ from the human response. For example, transfusion of hGPA RBCs typically results in no immune response and alloimmunization can only be induced in the presence of a strong inflammatory stimulus, such as poly I:C [87]. Transfusion of incompatible hGPA RBCs has been shown to result in clearance and a pro-inflammatory

cytokine storm, however this requires passive immunization of the recipients with anti-hGPA antibodies, in contrast to actively generated antibodies in the recipient from a prior hGPA exposure[149, 171]. Transfusion of HEL or HOD RBCs into an immunized recipient does not result in clearance of the incompatible red cells but instead results in selective removal of the HEL antigen [163, 164].

Our group recently sought to generate a new mouse model that expresses a clinically significant red cell antigen, and that can more closely mimic transfusion induced alloimmunization as it occurs in humans, with induction of a robust and boostable antibody response regardless of the presence or absence of inflammation, and resulting in a transfusion reaction due to antibody mediated clearance. This model expresses the Kell gene under the control of a β-globin promoter, which leads to red blood cell specific expression of the Kell glycoprotein. Initial studies confirmed that KEL was only expressed on red cells, with no expression on platelets, peripheral WBCs, or splenocytes. Also shown was that expression begins early in erythropoiesis, as is seen with human KEL expression, and remains stable for the entire circulatory life of the red cell[172].

SUMMARY

Though transfusion of blood has long been recognized for its importance in the treatment of many diseases, the field in general remains exceedingly understudied. The progress in understanding the immune responses to

transfused blood is in especially stark contrast to the related but much newer field of solid organ transplantation, for which the immune response is much better described, and many more prophylactic treatments have been developed. Improvements have undeniably been made in blood donation and collection, infectious disease testing, and storage and preparation of blood for transfusion, however the immune response to red cell antigens remains incompletely understood, and the prevention of immune-mediated complications of transfusion relies much too heavily on avoidance of exposure, which is sometimes not feasible, especially for those who require chronic transfusions. Red cell alloimmunization through pregnancy to any antigen other than RhD is also unpreventable and when it occurs can have serious and fatal consequences for the fetus.

Significant progress has been made in determining the factors involved in the immune response to solid organ transplantation using murine models, demonstrating their usefulness in studying alloimmunization. However, many differences exist between the characteristics of RBC and solid organ antigens, as well as the immune cells and organs they come in contact with, and thus it is probably that the generation and consequences of the alloimmune response differs as well. The murine models of transfusion associated alloimmunization that have been generated so far have provided insight into the mechanisms of both the afferent and efferent immune response to red cell antigens, but unfortunately, no models have yet been able to recapitulate the specific aspects of the human response to a clinically significant red cell antigen, demonstrating

the need for additional, improved models. We have recently developed a new transgenic mouse that has red blood cell specific expression of the human Kell antigen, which has been implicated in causing not only severe hemolytic transfusion reactions, but also hemolytic disease of the fetus and newborn. The following chapters describe our analysis of the generation of the immune response to the Kell RBCs, the resulting consequences of transfusion of these red cells into an immunized recipient, and also demonstrate how this model can also be used to investigate red cell alloimmunization through pregnancy.

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Chapter 2

Transfusion of murine RBCs expressing the human KEL glycoprotein induces clinically significant alloantibodies

ABSTRACT

Red blood cell (RBC) alloantibodies to non-self antigens may develop following transfusion or pregnancy, leading to morbidity and mortality in the form of hemolytic transfusion reactions or hemolytic disease of the newborn. A better understanding of the mechanisms of RBC alloantibody induction, or strategies to mitigate the consequences of such antibodies, may ultimately improve transfusion safety. However, such studies are inherently difficult in humans. We recently generated transgenic mice with RBC specific expression of the human KEL glycoprotein, with the KEL2 or KEL1 antigens. Herein, we investigate recipient alloimmune responses to transfused RBCs in this system. Transfusion of RBCs from KEL2 donors into wild type recipients (lacking the human KEL protein but expressing the murine KEL orthologue) resulted in dose dependent anti-KEL glycoprotein IgM and IgG antibody responses, enhanced by recipient inflammation with poly (I:C). Boostable responses were evident upon repeat transfusion, with morbid appearing alloimmunized recipients experiencing rapid clearance of transfused KEL2 but not control RBCs. Although KEL1 RBCs were also immunogenic following transfusion into wild type recipients, transfusion of KEL1 RBCs into KEL2 recipients or vice versa failed to lead to detectable anti-KEL1 or anti-KEL2 responses. This murine model, with reproducible and clinically significant KEL glycoprotein alloantibody responses, provides a platform for future mechanistic studies of RBC alloantibody induction and consequences. Long term translational goals of these studies include improving transfusion safety for at risk patients.

INTRODUCTION

RBC alloimmunization is a clinically significant problem that affects men, women, and children alike. These RBC alloantibodies may increase morbidity and morality, putting patients at risk for acute and hemolytic transfusion reactions, hemolytic disease of the fetus and newborn, or delays in locating compatible blood. In fact, hemolytic transfusion reactions due to non-ABO antibodies were the 2nd leading cause of transfusion associated death reported to the US FDA last year[1]. 1/600 pregnancies are affected by RBC alloantibodies, with some women entering pregnancy with RBC alloantibodies due to prior transfusions and others becoming alloimmunized during pregnancy or after delivery[2]. Thus, RBC alloantibodies can be dangerous in transfusion and pregnancy settings alike.

Outside of transfusion avoidance or limited phenotypic/genotypic matching, very few therapeutic strategies exist to prevent or to minimize the dangers of RBC alloimmunization. To date, much has been learned about the functional characteristics and immunogenicity of individual human RBC antigens[3,4]. However, the numbers of variables involved in each transfusion make studying factors influencing the formation of alloantibodies difficult. Such variables may include donor and recipient health status, donor and recipient genetics and antigenic differences, and donor unit preparation/storage. Furthermore, ethical and practical considerations limit in depth studies of the mechanisms of RBC alloantibody induction in humans. Lastly, human studies involving RBC

clearance patterns have historically been limited by the method (radioactivity) of RBC labeling, which allows a gross estimate of RBC clearance but limits in depth analyses of individual transfused RBCs.

The therapeutic void for prevention and treatment of RBC alloimmunization is due, in part, to a lack of existing experimental models. In fact, the mechanism of action of the sole targeted immunomodulatory therapy in existence (polyclonal anti-D or RhoGam) remains unknown[5,6]. Although a number of monoclonal anti-D preparations have been tested in humans[7,8], no animal model with RBC specific expression of the complex D antigen exists to delve in depth into potential mechanisms. With the realization that native murine RBC antigens[9,10] have not been thoroughly defined and are minimally immunogenic, our group has developed or worked with a number of transgenic murine models of RBC alloimmunization over the past decade[11,12]. Some have model antigen expression on RBCs (membrane bound hen egg lysozyme or mHEL)[13], others have human RBC antigen expression (human glycophorin or hGPA)[14,15] or Duffy^{b} [16], and yet others have a hybrid of model and human antigens (hen egg lysozyme, ovalbumin, Duffy^{b} or HOD) [17,18].

Each animal model has its own set of strengths and weaknesses; however, none recapitulates all aspects of a clinically significant human RBC antigen. The mHEL and Duffy^{b} models have ubiquitous antigen expression, something not found in many human RBC antigens; stringent removal of contaminating WBCs

and platelets is necessary for evaluation of RBC antigen responses. Anti-HEL antibodies generated in response to mHEL or HOD transfusions have been shown to result in selective removal of the HEL antigen instead of RBC clearance[19]; ongoing studies are further evaluating this phenomenon. The hGPA model results in anti-hGPA antibodies[20] when donors and recipients are MHC matched and when recipients are pre-treated with poly (I:C), though anti-hGPA (e.g. anti-M family) antibodies in humans are rarely clinically significant. Interestingly, hGPA transfusion in the absence of inflammation leads to non-responsiveness and potentially antigen specific tolerance[15]. Passively infused anti-hGPA antibodies have been shown to lead to clearance of antigen positive RBCs[21-24], with mechanistic studies ongoing. Thus, much has been learned and much remains to be learned from these models; however, none to date results in a reproducible, robust, and boostable alloantibody response in the absence of an adjuvant, leading to RBC clearance with clinically significant sequelae.

We sought to develop a new model of RBC alloimmunization with the above characteristics, ideally utilizing an existing human antigen. The “Kell factor” was initially described half a century ago, after the direct antiglobulin test was created and performed in instances following hydropic fetal complications[25]. Subsequently, anti-Kell family antibodies were shown to result in fatal hemolytic transfusion reactions[26]. Since that time, it has been shown that the Kell factor is actually a family of antigens, with Kell being a glycoprotein with endopeptidase activity[4]. Multiple epitopes on the Kell protein have been defined as clinically

significant antigens, including $Js^{a/b}$ and $Kp^{a/b27}$. The most well known, however, are KEL1 (also known simply as Kell) and KEL2 (also known as Cellano). Approximately 10% of transfusions are mismatched for KEL1/KEL2, with anti-KEL1 as well as anti-KEL2 antibodies leading to hemolysis of incompatible transfused RBCs or of incompatible fetal RBCs. In fact, KEL alloantibodies are a leading cause of transfusion and pregnancy associated morbidity/mortality today[28-33].

Herein, we describe recipient immune responses to our newly generated murine KEL model. This model involves the human KEL antigen expressed in an RBC specific fashion on murine RBCs[34]. Despite some homology between murine and human KEL[35], nearly all wild type C57BL/6 recipients of KEL2 RBCs form anti-KEL glycoprotein antibodies after a single transfusion. This response is boostable upon repeat exposure, enhanced in the presence of recipient inflammation with poly (I:C), and clinically significant in that anti-KEL glycoprotein antibodies lead to clearance of antigen positive RBCs. However, antibody generation appears limited to recipients that lack the human KEL antigen altogether, with animals with RBCs expressing an antithetical antigen (e.g. KEL2 RBCs into KEL1 recipients and vice versa) failing to develop detectable anti-KEL2 or anti-KEL1 alloantibodies under the conditions tested. Taken together, these findings lay the groundwork for in depth analyses of factors influencing RBC alloantibody induction as well as targeted immunomodulatory therapies to prevent the formation or to mitigate the dangers of RBC alloantibodies.

MATERIALS AND METHODS

Mice: C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD); KEL2 and KEL1 transgenic mice expressing the human KEL glycoprotein (previously published as "KEL2B" and "KEL1A") were generated in our laboratory [34] and bred at Emory. All animals were housed in the Emory University Department of Animal Resources facilities, and all procedures and protocols were approved by the Emory University Institutional Animal Care and Use Committee.

Murine blood collection, fluorescent labeling, and transfusion: RBCs were collected into acid-citrate-dextrose (ACD) and washed 3 times with phosphate buffered saline (PBS) to remove residual citrate. Anti-KEL2 (Clone LKL1, Alba, Edinburgh, UK) or anti-KEL1 (Mima 23, generously provided by Greg Halverson of the New York Blood Center) were utilized to confirm the presence of the KEL2 or KEL1 antigens on transfused RBCs. KEL2 RBCs were transfused in titrated amounts (0.5, 5, and 50 µL of RBCs, diluted in PBS to 300 µL total volume) via lateral tail vein; in some experiments, recipient animals were transfused every 2 weeks and in others, recipients were pretreated with 100 µg of poly (I:C) (Amersham, Piscataway, NY) given intraperitoneally (i.p.) 2 hours prior to transfusion. In a subset of experiments, RBCs were labeled with chloromethylbenzamido 1,1'- dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI) or 3,3'-dihexadecyloxacarbocyanine perchlorate (DiO) according to the manufacturer's instructions as previously described (Molecular

Probes, Eugene OR)[24,36,37]. After labeling, experimental and control RBCs were mixed at a 1:1 ratio and recipient animals were transfused via lateral tail vein with 50-75 µL of each blood type, diluted in PBS to 300 µL total volume.

Human RBC assays: Human RBCs from residual segments were tested for KEL1 and KEL2 expression by flow cytometry, using monoclonal reagents. Those with KEL2 but not KEL1 expression were selected, and some RBCs were treated with 0.2 M dithiothreitol (DTT). These DTT treated and untreated human KEL2 RBCs (alongside DTT treated and untreated transgenic murine KEL2 RBCs) were then utilized as targets for flow cytometric crossmatch with sera from alloimmunized mice.

Flow cytometry: Serum was analyzed for the presence of anti-KEL IgM and IgG utilizing indirect immunofluorescence (flow cytometric crossmatch) with KEL2, KEL1, or control C57BL/6 RBCs; an adjusted mean fluorescence intensity (MFI) was calculated by subtracting the signal of serum crossmatched with antigen negative targets from that of serum crossmatched with antigen positive targets. Transfused RBCs were analyzed for presence of bound IgM and IgG using APC-conjugated goat anti-mouse IgM and Alexafluor®488-conjugated goat anti-mouse IgG, respectively (Jackson Immunoresearch, West Grove PA), after gating on the lipophilically labeled RBCs. Post-transfusion KEL2 RBC recovery and survival were determined by flow cytometry and lipophilic dye fluorescence, utilizing a ratio of transfused KEL2 RBCs to control C57BL/6 RBCs. All

antibodies were used at 1:100 dilution and all samples were analyzed on a 4-color BD FACSCalibur.

Cytokine Analysis: Serum cytokines, including interleukin (IL)-6, keratinocyte-derived chemokine (KC, the murine equivalent to IL-8 in humans), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, TNF-α and IFN-γ, were evaluated 90-120 minutes post transfusion using the Cytometric Bead Array Mouse Flex Kit (BD Biosciences, San Diego, CA)

Statistical Analysis: Statistical significance was determined by performing a Students t-test for comparison of 2 groups or one-way analysis of variance (ANOVA) with Tukey's posttest when 3 groups were compared using Graphpad Prism software (San Diego, CA). Error bars represent one standard deviation and differences were considered statistically significant with p-values ≤0.05.

RESULTS

Serologic responses of C57BL/6 recipients to transfused KEL2 RBCs

Blood was collected from KEL2 donors, and the presence of KEL2 expression on the pooled RBCs was determined prior to transfusion using monoclonal anti-KEL2 (**Figure 2.1A**). The equivalent of 1 human "unit" of KEL2 RBCs (50-75 μL) was then transfused into wild type C57BL/6 recipients. 14-21 days post-transfusion, recipient serum was crossmatched with KEL2 or wild type control C57BL/6 RBC targets, using anti-mouse IgG as a secondary reagent (**Figure 2.1B** shows a representative crossmatch after 3 transfusions). An adjusted mean fluorescence intensity (MFI) was calculated by subtracting the background signal of serum crossmatched with wild type RBCs from that of serum crossmatched with KEL2 RBCs. The only known difference between wild type C57BL/6 and KEL2 RBC targets is the KEL glycoprotein antigen[34], thus eliminating MHC mismatch as a variable and leading one to hypothesize that the observed adjusted MFI signal is due to anti-KEL glycoprotein alloantibodies.

To further evaluate the specificity of the anti-KEL glycoprotein antibody response, serum from immunized mice was crossmatched with murine KEL2 RBCs or murine KEL2 RBCs treated with 0.2M dithiothreitol (DTT) (**Figure 2.1C**). DTT is known to reduce disulfide bonds to free sulfhydryl groups, with disulfide bonds being a requirement for KEL antigen integrity[38]. These serum crossmatches were repeated using human RBCs expressing the KEL2 antigen or human RBCs expressing the KEL2 antigen that had been treated with 0.2M DTT as targets

(**Figure 2.1D**). In both instances, the observed reactivity with intact KEL2 expressing murine or human target RBCs was essentially eliminated upon crossmatch with DTT treated target RBCs. These results lend further support to the KEL specificity of the alloantibody observed in the sera of C57BL/6 recipients following transfusion with transgenic murine KEL2 RBCs.

In order to evaluate dose responses to transfused KEL RBCs, 0.5, 5, or 50 µL of KEL2 RBCs were transfused into C57BL/6 recipients, with serum evaluated 2 weeks later by flow crossmatch with transgenic murine KEL2 or C57BL/6 RBCs. In 2/2 experiments (n=27 animals total), a dose titratable anti-KEL glycoprotein IgG response was observed (**Figure 2.2A** shows adjusted MFI). For the remainder of the experiments described herein, 50 µL of RBCs were thus utilized for transfusion. To more completely characterize the immune response of C57BL/6 recipients to transfused KEL2 RBCs, wild type or KEL2 syngeneic recipients were transfused with 50 µL of KEL2 RBCs, and anti-KEL IgM and IgG in the serum was measured by flow crossmatch from days 3 through 28 post-transfusion. In a compilation of 3 independent experiments (n=24 animals total), anti-KEL IgM was observed in the serum of C57BL/6 but not syngeneic recipients by day 3 post-transfusion, peaking by day 5. Anti-KEL IgG was observed in the serum of C57BL/6 but not syngeneic recipients by day 7 post-transfusion, peaking near days 14-21. (**Figure 2.2B-C** shows a representative experiment).

Assessment of repeat antigen exposure and and cytokine responses

Given the reported evanescence patterns of RBC alloantibodies in humans[39,40], antigen positive RBCs may be unintentionally transfused into humans with alloantibodies that fall below the level of detection by traditional blood bank antibody screening methodologies. Anamnestic responses may subsequently result, with a rapid increase in RBC alloantibody titer and premature clearance of transfused RBCs. In fact, some such transfusions may lead to such significant hemolysis of both transfused and “by-stander” RBCs that renal failure, DIC, and death result[41]. Past efforts by our laboratory to create murine models with antibody responses that are "boostable" in the absence of co-existent recipient inflammation have thus far not been successful, likely due to characteristics of the antigens previously studied. To examine potential boostable responses in our newly developed KEL system, KEL2 RBCs were transfused every 2 weeks for a total of 3 transfusions into C57BL/6 recipients, with total anti-KEL Igs measured 2 weeks after each transfusion. In 3/3 experiments (n=15 recipients total), significant increased responses were observed following each transfusion (**Figure 2.3A** shows a representative experiment**)**. This increased alloantibody response was not simply due to a continued increase in anti-KEL titer over time, as control animals receiving a single transfusion had peak alloantibody responses at approximately 14-21 days post-transfusion.

In humans, incompatible RBC transfusions are the 2nd leading cause of transfusion associated death[1]. To date, however, few animal models have been created in which repeat transfusions not only lead to high titer alloantibodies but

also to adverse clinical outcomes. Two laboratories have reported an animal model in which anti-hGPA antibodies resulted in clearance of incompatible RBCs and a pro-inflammatory serum cytokine storm[21-24,36]; however, these antibodies were passively infused and not actively generated. In our current studies, C57BL/6 recipients were actively alloimmunized through repeat KEL2 transfusions. These alloimmunized recipients were then re-transfused a 4th time with "incompatible" KEL2 RBCs and observed. Within 5 minutes of the incompatible transfusion, recipients appeared ill and hunched over. Cytokine bead arrays were performed on recipient serum 90-120 minutes post-transfusion, revealing statistically significant elevations of IL-6, KC, MCP-1, MIP-1B, and TNF-α in alloimmunized animals receiving "incompatible" RBCs; no changes were observed in IFN-γ (**Figure 2.3B-F** shows representative data).

Anti-KEL responses in the presence of recipient inflammation with poly (I:C)

It is known that a "danger" signal of sorts leads to an augmented immune response in a number of settings, both clinical and experimental[42]. We have previously reported that recipient inflammation with the double stranded RNA poly (I:C) enhances RBC alloimmunization in multiple different murine RBC alloimmunization models, including the mHEL, hGPA, and HOD models[15,43,44]. To evaluate the effect of recipient inflammation on alloimmunization in our KEL model, C57BL/6 recipient animals were treated with 100 μg of poly (I:C) i.p.; control animals were treated with PBS i.p. Subsequently, all recipients were

transfused with KEL2 RBCs and serum alloantibody responses were evaluated at multiple time points post-transfusion. In 3/3 experiments (n=27 animals total), poly (I:C) treated animals had significantly higher serum anti-KEL IgG levels post-transfusion than control animals (**Figure 2.4A** shows compilation data on day 14 post-transfusion, $p<0.05$). To determine boostable responses in the presence of poly (I:C), 3 separate cohorts of C57BL/6 recipients were transfused 1, 2, or 3 times in the presence of poly (I:C). Serum samples were evaluated every 2 weeks in each cohort, out 42 days from the time of the initial transfusion. Similar to what was observed in the absence of poly (I:C), boostable responses were observed in the presence of poly (I:C) with peak titers noted approximately 2 weeks post-transfusion (**Figure 2.4B** shows representative data). These responses were not an artifact of increased anti-KEL responses over time, as control animals receiving a single transfusion did not have significant increases in anti-KEL beyond 14-21 days post-transfusion (**Figure 2.4B** shows 3 distinct cohorts of animals receiving 1, 2, or 3 transfusions, with anti-KEL glycoprotein antibody responses measured over time in each cohort).

Anti-KEL RBC binding and clearance

The studies described to this point have focused on serologic evaluation of transfused recipients. Serum is the focus of clinical transfusion medicine evaluations, given difficulties in evaluating antibody bound to transfused RBCs. In fact, “mixed field” reactions, in which a subset of recipient RBCs (e.g. those

transfused) have bound antibody whereas another subset of recipient RBCs (e.g. the patient's own RBCs) lack bound antibody, can be quite technically challenging to evaluate. Practically speaking, however, an evaluation of the RBC antibody bound in vivo may be a better predictor of the fate of the transfused RBCs than an in vitro test of serum alloantibodies.

To allow visualization of cells in the recipient post-transfusion, KEL2 RBCs were labeled before transfusion with a lipophilic dye (DiI) that intercalates into the lipid RBC membrane; control C57BL/6 RBCs were labeled with a dye that fluoresces on a different channel (DiO). These dyes have been carefully dose titrated, such that the RBC circulatory half life remains unaffected[34]. KEL2 and C57BL/6 RBCs were mixed and transfused. Direct antiglobulin tests (DAT) for IgM and IgG were performed at multiple time points post-transfusion on transfused DiI positive KEL2 RBCs, comparing signal on transfused DiI positive KEL2 RBCs to that of control C57BL/6 RBCs (**Figure 2.5A-B**). In 3/3 experiments (n=24 animals total), anti-KEL glycoprotein IgM and IgG were detected on circulating KEL2 RBCs by approximately days 3 and 7, respectively. The timing of the initial detection of anti-KEL bound to circulating RBCs thus closely parallels that of anti-KEL detected in serum.

Transfused recipients were also serially evaluated post-transfusion for clearance of KEL2 RBCs. A ratio of DiI positive KEL2 RBCs to DiO positive C57BL/6 RBCs was determined at multiple time points post-transfusion (**Figure 2.5C**) in

syngeneic KEL2 recipients or wild type naïve C57BL/6 recipients. Although little to no detectable KEL specific RBC clearance was observed in syngeneic KEL2 recipients to which KEL2 is a self-antigen, a slow relative decline in the ratio of transfused KEL2 to C57BL/6 RBCs was appreciated in wild type C57BL/6 recipients over a 28 day period (**Figure 2.5D** depicts a representative experiment with 3-5 animals/group).

Given the morbid appearance and serum cytokine storm observed in alloimmunized animals transfused with "incompatible" KEL2 RBCs, we hypothesized that KEL specific RBC clearance would rapidly occur. To test this hypothesis, alloimmunized or naïve C57BL/6 recipients were transfused with DiI labeled KEL2 and DiO labeled C57BL/6 RBCs, with post-transfusion KEL2 RBC recovery and survival tracked. As hypothesized, the "incompatible" KEL2 RBCs were cleared more rapidly post-transfusion in alloimmunized than naïve C57BL/6 recipients (**Figure 2.5E** depicts a representative experiment with 3-5 animals/group). Furthermore, recipients who were initially treated with poly (I:C) and transfused, with demonstrable higher titer anti-KEL glycoprotein alloantibodies, had subtly higher rates of KEL specific RBC clearance (**Figure 2.5E**). This clearance was not an artifact of decreased stability of the KEL2 RBCs in general, however, as these RBCs had a normal circulatory half-life in syngeneic KEL2 recipients.

Serum responses to single amino acid polymorphisms on murine RBCs

Although some human RBC antigens (such as D) may be present on donor RBCs and absent on recipient RBCs, other human antigens differ by just a single amino acid polymorphism to their "antithetical" antigen. To test the immunologic effect of crossing this small antigenic barrier in mice, a separate strain of animals was created with RBC specific expression of the human KEL glycoprotein expressing the KEL1 antigen[34] (also known as "Kell"). Unlike the robust anti-KEL glycoprotein response observed after 3 transfusions in wild type C57BL/6 recipients, no anti-KEL2 IgG could be detected in KEL1 recipients (**Figure 2.6A**). Furthermore, transfusions in the presence of poly (I:C) also failed to lead to detectable anti-KEL2 responses in KEL1 recipients.

Additional studies were completed using KEL1 RBCs transfused into KEL2 recipients, which is the scenario most likely to be encountered in human clinical setting. We have previously shown stable KEL1 antigen expression, epitope integrity, and post-transfusion recovery/survival in these animals[34]; **Figure 2.6B** shows KEL1 transgenic RBCs stained with a monoclonal anti-KEL1 reagent (Mima 23, generously provided by Greg Halverson of the New York Blood Center). KEL1 RBCs were transfused every 2 weeks x 3 into KEL2 recipients, in the presence of poly (I:C). As described in the KEL2 donor/KEL1 recipient setting, the KEL1 donor/KEL2 recipient setting also failed to generate detectable serum anti-KEL1 responses (**Figure 2.6C**). This lack of a response was not due to KEL1 RBCs being incapable of being immunogenic, as wild type C57BL/6

recipients made detectable anti-KEL glycoprotein antibodies after KEL1 RBC transfusion (**Figure 2.6D**).

DISCUSSION

Herein, we have described our initial observations of alloimmune responses to transfused RBCs from newly generated transgenic donors expressing a human RBC antigen. These donors have RBC specific expression of the KEL glycoprotein[34], which makes up a blood group of antigens in humans. The KEL glycoprotein in these animals has intact expression of multiple epitopes found in humans, including Js^b, Kp^b, and KEL2 (also known as Cellano)[34]. KEL2 RBCs transfused into C57BL/6 animals, which lack the human KEL antigen altogether, result in robust, dose titratable, and boostable anti-KEL glycoprotein IgM and IgG responses, which are further enhanced by recipient inflammation with the double stranded RNA poly (I:C).

"Responder" rates of KEL alloimmunization approach 100% in this model, presumably due in part to the degree of donor/recipient RBC antigenic differences. The model somewhat resembles that of D in humans, in that donors may express the D antigen which recipients lack altogether. It should be noted that murine KEL is approximately 75% orthologous to human KEL at the amino acid level[35], and thus the human KEL glycoprotein is not altogether foreign to the murine recipients. Given that very few humans actually lack the KEL glycoprotein, additional transgenic animals were generated to test the impact on RBC alloimmunogenicity of a single amino acid polymorphism between donor and recipient. Both KEL2 and KEL1 animals have RBC specific expression of the human KEL glycoprotein, with a single amino acid polymorphism difference.

Unlike the high responder rates observed when a relatively large antigenic barrier (KEL2 into wild type C57BL/6) was crossed, no antibody responses to date have been detected with KEL2 RBCs transfused into KEL1 recipients or vice versa. It is unclear if KEL1 or KEL2 mice are capable of presenting the polymorphic peptide between KEL1 and KEL2 in their MHC II; as C57BL/6 animals have a single MHC II molecule, this is a distinct possibility. Ongoing studies are continuing to investigate KEL1/KEL2 responses, including MHC diversity analyses.

In addition to studies of immunity induction, this KEL model allows for studies of the fate of transfused RBCs. Human studies utilizing chromium labeling are somewhat limited to blunt evaluations of clearance curves. The strategy of labeling RBCs with a lipophilic dye, as previously described by our group, allows for in depth flow cytometric evaluation of the transfused cells. A slow but reproducible pattern of KEL2 specific RBC clearance was noted following an initial transfusion, with more rapid clearance and a pro-inflammatory serum cytokine response observed in previously alloimmunized recipients with higher titer alloantibodies. The degree of intravascular versus extravascular hemolysis cannot be evaluated from the current studies, as extensive blood or urine chemistry analyses were not completed. However, there was no appreciable “by-stander” hemolysis of the recipient’s own RBCs, as hematocrit values were noted to be similar in alloimmunized and non-alloimmunized recipients of KEL2 RBCs (data not shown). Clearance patterns correlated with detectable serum

and RBC bound anti-KEL Igs, with no clearance of antigen negative C57BL/6 RBCs noted. However, in no experiment were all transfused KEL2 RBCs cleared by day 28. These findings suggest that KEL2 RBCs remaining in circulation may be acquiring a survival advantage of sorts. We have previously reported RBC populations resistant to hemolysis in the hGPA system[36]. In addition, KEL antigens are known to have the potential to undergo weakened antigenicity during incompatibility in human settings[45,46]; ongoing studies are investigating clearance as well as clearance resistance mechanisms in our model.

Limitations to the KEL into C57BL/6 model and experiments described must be considered. One such consideration is that this model resembles that of D in humans more so than that of KEL1/KEL2, in that the human KEL antigen is present on donor RBCs but lacking on recipient RBCs. This is, perhaps, more of a deviation from what is “human” than a true scientific limitation of the model; findings from this described model will likely have applicability to some but not all antigens. Other considerations include the potential confounders involved in utilizing lipophilic dyes to track transfused RBCs; despite careful titration and control studies, their impact on study results cannot definitively be ruled out. Another consideration is that poly (I:C) is just one type of recipient inflammation, and other toll like receptor agonists[43] or authentic infections may have differential effects on alloimmunization outcomes. A last consideration is that these studies were completed utilizing KEL2 and KEL1 animals which have approximately 1200 and 800 copies of KEL glycoprotein per RBC[34]. Additional KEL transgenic

animals have since been created with lower and higher RBC antigen copy numbers, with ongoing studies investigating the impact of copy number and dose responses on alloimmunogenicity and clinical consequences.

In sum, the KEL model described herein offers a reductionist system in which to better understand both the induction of KEL specific RBC alloantibodies as well as their clinical consequences. A number of questions remain unanswered, and much work remains to be completed. However, this model, with a boostable, clinically significant alloantibody response, will serve as a platform for the investigation of immunomodulatory strategies to prevent or mitigate RBC alloantibody formation as well as subsequent sequelae. An improvement in the understanding and treatment of RBC alloimmunization, in a bench to bedside and back manner, remains a necessity in improving transfusion safety in men, women, and children alike.

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Figure 2.1. Transgenic murine RBCs express the human KEL2 antigen, and C57BL/6 recipients of KEL2 RBC transfusion make alloantibodies with KEL specificity. (**A**) Murine RBCs from KEL2 transgenic donors were collected and stained with anti-KEL2 prior to transfusion. (**B**) Serum from C57BL/6 recipients transfused with KEL2 RBCs was crossmatched with murine KEL2 RBCs (solid line) or wild type C57BL/6 RBCs (shaded histogram). (**C**) Serum from C57BL/6 recipients transfused with KEL2 RBCs was crossmatched with murine KEL2 RBCs (solid line) or with DTT treated murine KEL2 RBCs (shaded histogram). (**D**) Serum from C57BL/6 recipients transfused with KEL2 RBCs was crossmatched with human RBCs expressing KEL2 (solid line) or with DTT treated human RBCs (shaded histogram); representative results are shown.

Figure 2.2. C57BL/6 recipients lacking human KEL have a dose dependent anti-KEL glycoprotein antibody response. (**A**) Control (KEL2) or wild type C57BL/6 recipients were transfused with 0.5, 5, or 50 μL of KEL2 RBCs, with serum anti-KEL glycoprotein IgG evaluated 2 weeks post-transfusion. Serial evaluations of serum anti-KEL glycoprotein IgM (**B**) or IgG (**C**) were completed in KEL2 or wild type C57BL/6 recipients after a single transfusion of 50 μL of KEL2 RBCs. Results are representative of 2-3 independent experiments with at least 3-5 mice/group; *$p<0.05$.

Figure 2.3. C57BL/6 recipients have "boostable" responses to repeat KEL2 exposure, with a proinflammatory serum cytokine storm. (**A**) C57BL/6

recipients were transfused every 2 weeks with KEL2 RBCs, with serum anti-KEL glycoprotein Igs evaluated on day 14 after each transfusion. (**B-F**) Serum cytokine responses in alloimmunized animals, 90-120 minutes after a 4th KEL2 RBC transfusion. Results are representative of 2-3 independent experiments with at least 3-5 mice/group; *p<0.05.

Figure 2.4. Recipient inflammation with poly (I:C) enhances anti-KEL responses, with "boostable" responses. (**A**) Serum anti-KEL glycoprotein IgG responses in C57BL/6 animals transfused with KEL2 RBCs in the presence or absence of recipient poly (I:C) pretreatment; straight serum, 14 days post-transfusion. (**B**) Serum anti-KEL glycoprotein IgG responses after 1, 2, or 3 KEL2 RBC transfusions in the presence of poly (I:C); sera diluted 1:10, tested every 14 days. Results are representative of 2-3 experiments with 3-5 mice/group; *p<0.05

Figure 2.5: Circulating anti-KEL glycoprotein Igs bind to transfused KEL2 RBCs and are associated with KEL2 RBC clearance. KEL2 and C57BL/6 RBCs were labeled with DiI and DiO, respectively, prior to transfusion. RBC bound IgM (**A**) and IgG (**B**) were evaluated serially post-transfusion on DiI positive KEL RBCs; shaded histograms are control antigen negative cells. Post-transfusion survival and recovery of KEL2 RBCs was determined by comparing a ratio of DiI KEL to DiO C57BL/6 RBCs (**C**). These studies were completed KEL2 and wild type C57BL/6 recipients (**D**); similar studies were also completed

following a 2nd KEL2 transfusion in recipients initially transfused with or without poly (I:C) (**E**). Error bars represent standard deviation and results represent at least 3 independent experiments with 3-5 mice/group.

Figure 2.6: No detectable alloimmune responses to KEL2 RBCs occur in KEL1 recipients (or vice versa), whose KEL RBC antigens differ by a single amino acid polymorphism. (**A**) Serum responses of KEL1 or C57BL/6 recipients after 3 transfusions of KEL2 RBCs RBCs. (**B**) KEL1 RBCs were stained with monoclonal anti-KEL1 (Mima 23) prior to transfusion. Representative serum response of KEL2 (**C**) or C57BL/6 (**D**) recipients after 3 transfusions with KEL1 RBCs. Results are representative of 2-3 independent experiments with 2-5 mice/group; *$p<0.05$.

Figure 2.1

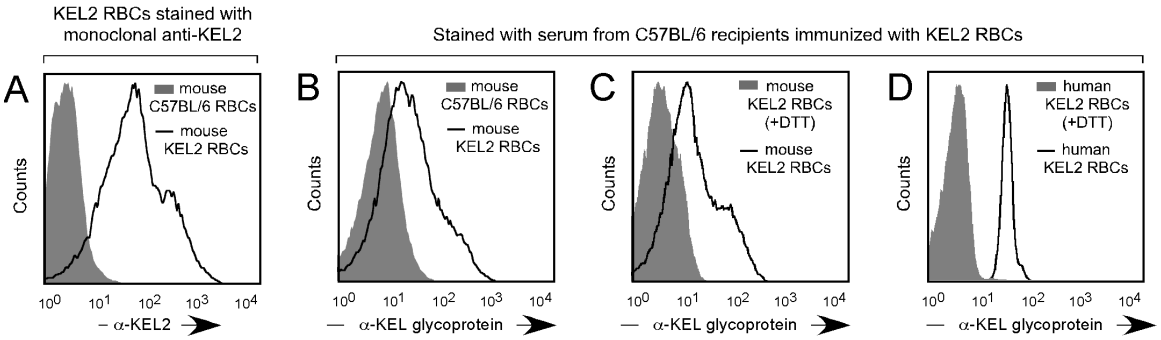

KEL2 RBCs stained with monoclonal anti-KEL2
Stained with serum from C57BL/6 recipients immunized with KEL2 RBCs
A
B
C
D
Counts
mouse C57BL/6 RBCs
mouse KEL2 RBCs
mouse KEL2 RBCs (+DTT)
human KEL2 RBCs (+DTT)
human KEL2 RBCs
10^0 10^1 10^2 10^3 10^4
– α-KEL2
— α-KEL glycoprotein

Figure 2.2

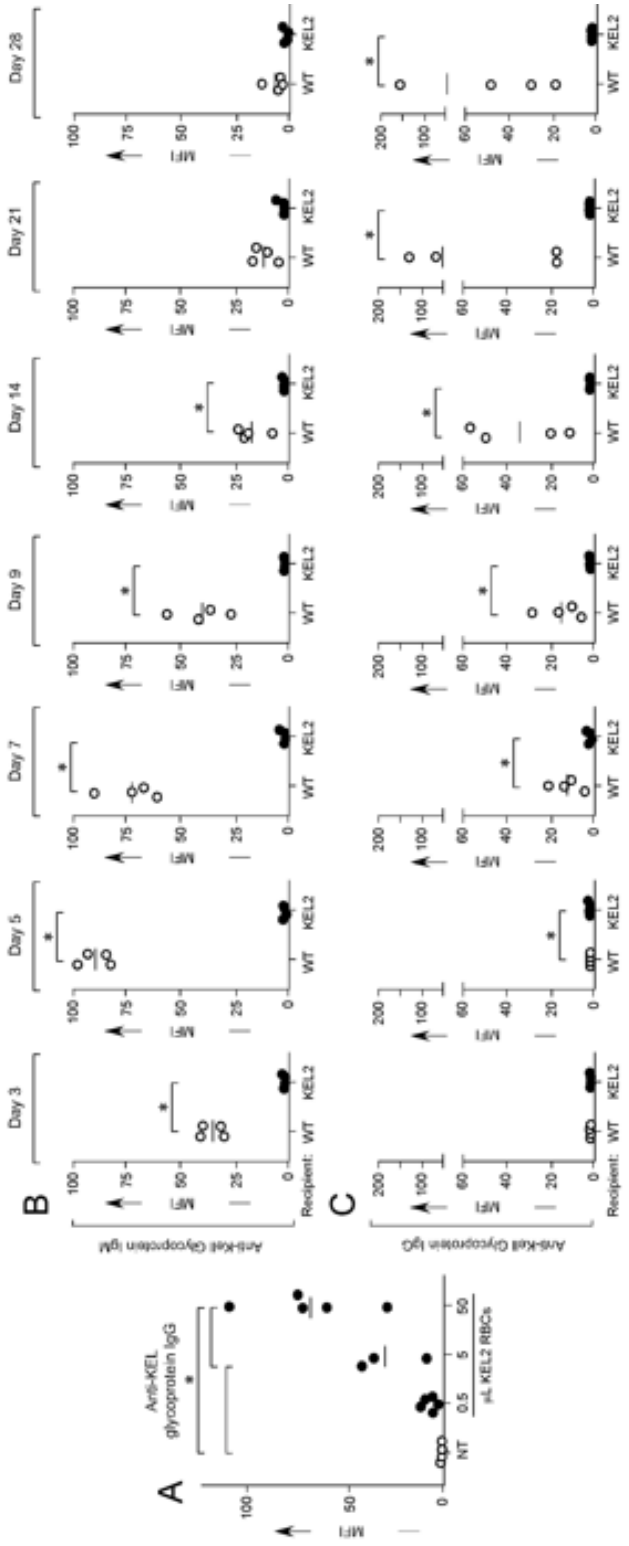

A
Anti-KEL glycoprotein IgG
MFI
NT
0.5
5
50
mL KEL2 RBCs
B
Anti-Kell Glycoprotein IgM
Day 3
Day 5
Day 7
Day 9
Day 14
Day 21
Day 28
Recipient:
WT
KEL2
C
Anti-Kell Glycoprotein IgG

Figure 2.3

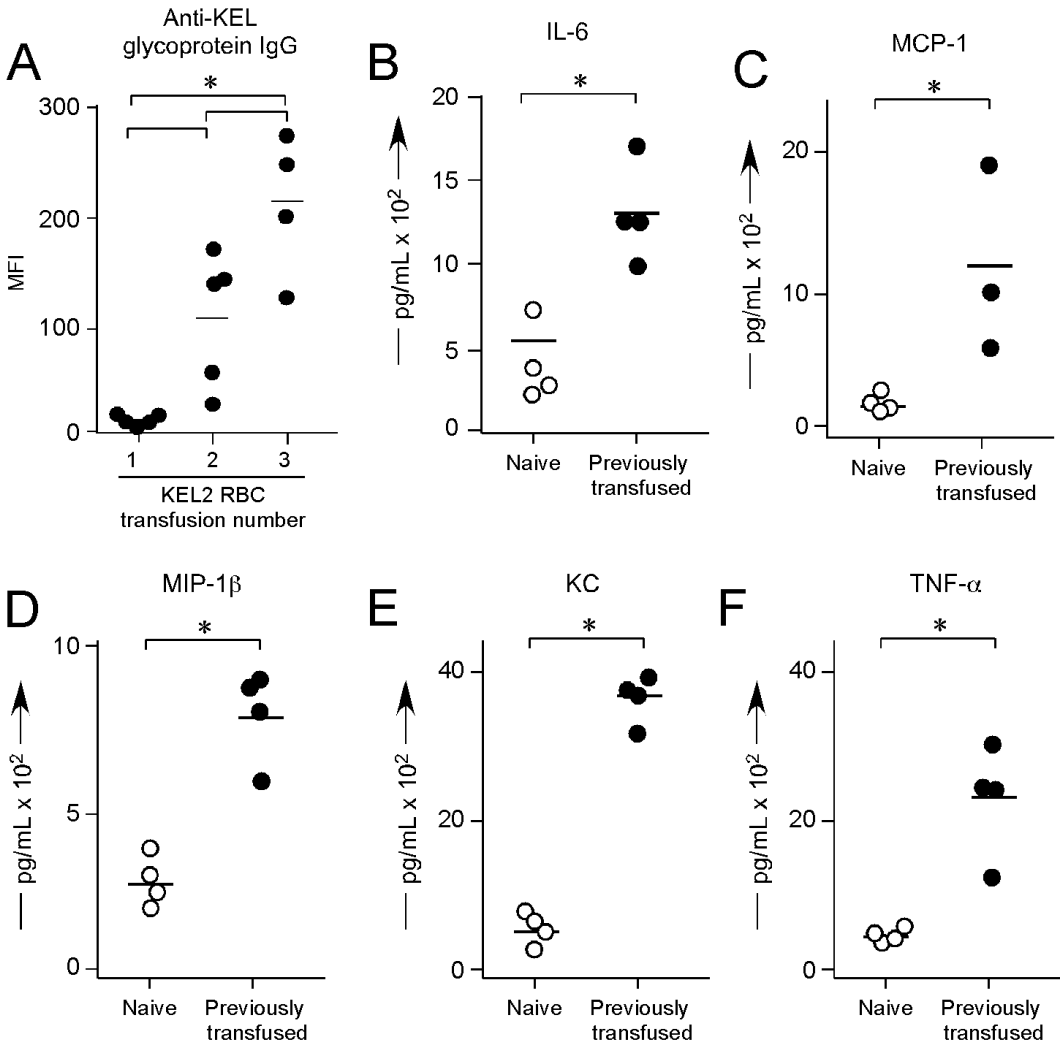

A
Anti-KEL glycoprotein IgG
MFI
300
200
100
0
1
2
3
KEL2 RBC transfusion number
B
IL-6
pg/mL x 10²
20
15
10
5
0
Naive
Previously transfused
C
MCP-1
pg/mL x 10²
20
10
0
Naive
Previously transfused
D
MIP-1β
pg/mL x 10²
10
5
0
Naive
Previously transfused
E
KC
pg/mL x 10²
40
20
0
Naive
Previously transfused
F
TNF-α
pg/mL x 10²
40
20
0
Naive
Previously transfused

Figure 2.4

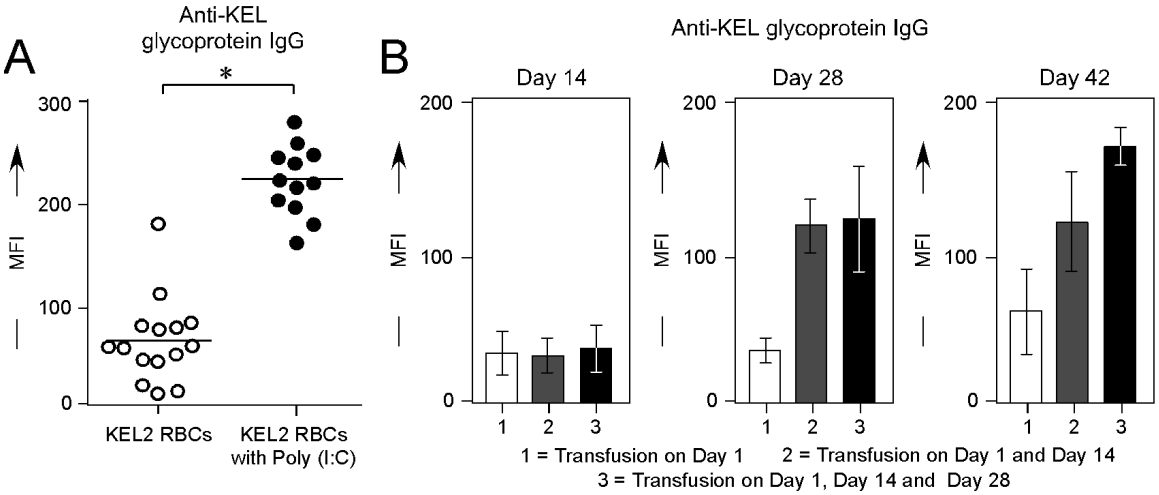

A
Anti-KEL glycoprotein IgG
*
MFI
300
200
100
0
KEL2 RBCs
KEL2 RBCs with Poly (I:C)
B
Anti-KEL glycoprotein IgG
Day 14
Day 28
Day 42
MFI
200
100
0
1
2
3
1 = Transfusion on Day 1
2 = Transfusion on Day 1 and Day 14
3 = Transfusion on Day 1, Day 14 and Day 28

Figure 2.5

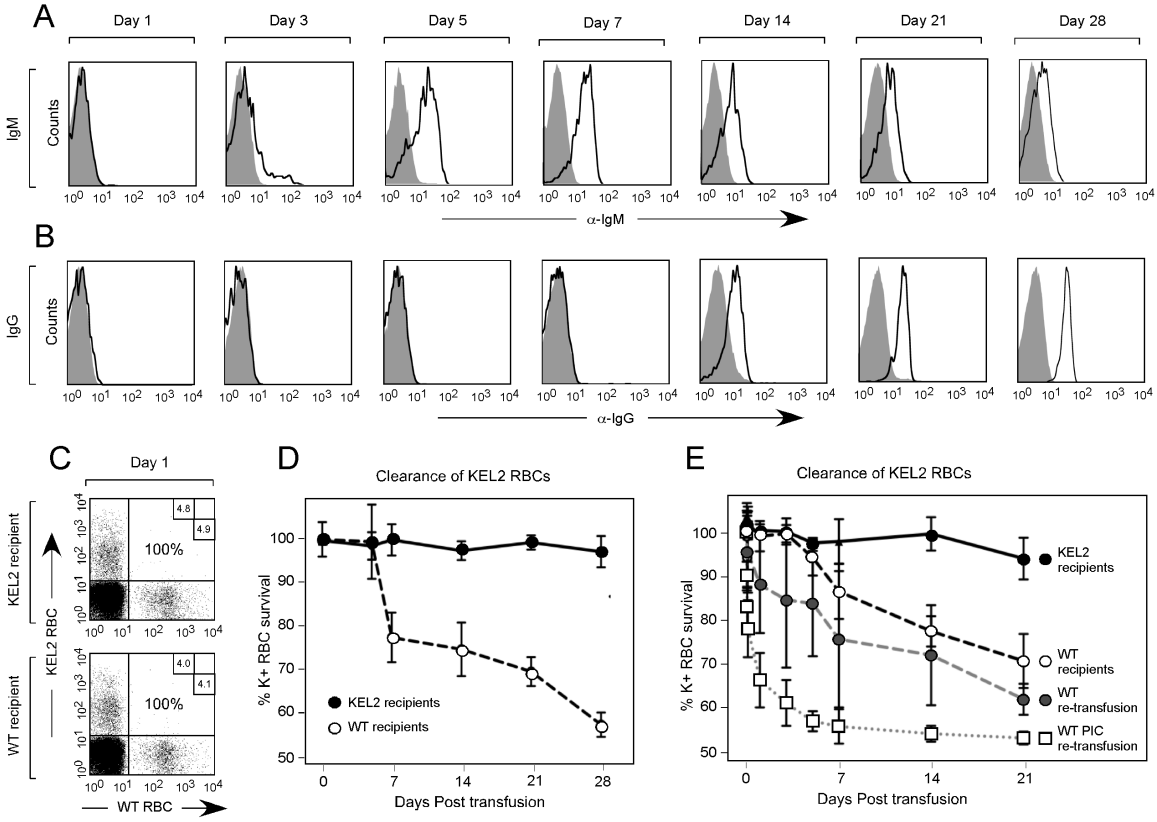
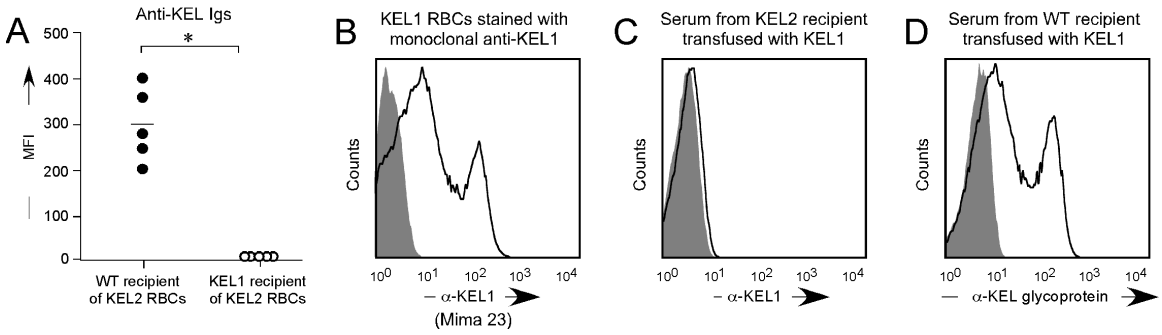

A
Day 1
Day 3
Day 5
Day 7
Day 14
Day 21
Day 28
IgM
Counts
α-IgM
B
IgG
Counts
α-IgG
C
Day 1
KEL2 recipient
WT recipient
KEL2 RBC
WT RBC
4.8
4.9
100%
4.0
4.1
100%
D
Clearance of KEL2 RBCs
% K+ RBC survival
KEL2 recipients
WT recipients
Days Post transfusion
E
Clearance of KEL2 RBCs
% K+ RBC survival
KEL2 recipients
WT recipients
WT re-transfusion
WT PIC re-transfusion
Days Post transfusion

Figure 2.6


A
Anti-KEL Igs
*
MFI
500
400
300
200
100
0
WT recipient of KEL2 RBCs
KEL1 recipient of KEL2 RBCs
B
KEL1 RBCs stained with monoclonal anti-KEL1
Counts
10^0 10^1 10^2 10^3 10^4
– α-KEL1
(Mima 23)
C
Serum from KEL2 recipient transfused with KEL1
Counts
10^0 10^1 10^2 10^3 10^4
– α-KEL1
D
Serum from WT recipient transfused with KEL1
Counts
10^0 10^1 10^2 10^3 10^4
— α-KEL glycoprotein

Chapter 3

Depletion of marginal zone B cells inhibits red blood cell alloimmunization

ABSTRACT

Similar to transplantation, transfusion of red blood cells (RBCs) can represent a life saving intervention. However, patients requiring repeat transfusion often develop RBC specific alloantibodies that decrease the therapeutic efficacy of transfused cells and limit the availability of compatible RBCs for future transfusion. Unlike transplantation, no prophylactic immunosuppression exists to inhibit RBC alloimmunization. We sought to elucidate the key factors responsible for RBC alloimmunization in an effort to develop a strategy to inhibit these effects. In efforts to achieve this, we use a novel transgenic model that specifically expresses the KEL glycoprotein on murine RBCs, and has been shown to induce robust anti-KEL antibody responses in KEL negative recipients capable of clearing Kell positive RBCs and inducing rapid removal and a systemic inflammatory response following subsequent transfusion. In this study, we show that removal of the spleen and more particularly, depletion of marginal zone B cells within the spleen, prevented IgG alloimmunization and immunological clearance of Kell positive RBCs in Kell negative recipients. Furthermore, marginal zone B cell depletion prior to primary Kell positive RBC exposure prevented the rapid clearance and systemic inflammatory response normally observed following subsequent Kell positive RBC exposure. These results suggest an unprecedented role of marginal zone B cells in RBC alloimmunization and provide a unique therapeutic target to prevent RBC alloimmunization in chronically transfused individuals.

INTRODUCTION

Although adaptive immunity provides the ability to respond to evolving antigenic determinants, occasionally immunity targets innocuous antigens. Indeed, the most significant barrier to tissue transfer between individuals reflects immunological recognition of unique antigenic variations between donor and recipient[1-4]. While attempts to match donor and recipient antigens reduce immune-mediated rejection, perfect matches rarely occur[5]. As a result, individuals receiving donor tissue often undergo immunosuppression prior to and following engraftment to reduce immune recognition and destruction of donor tissue[6].

While immunosuppression precedes solid organ transfer, a similar prophylactic intervention does not typically occur prior to transfusion of red blood cells (RBCs). As RBC transfusion does not reflect a permanent donor tissue engraftment, rapid therapeutic increases in RBC mass can be achieved without actively inhibiting alloimmunization prior to and following transfusion. However, as RBC transfusion does not result in long-term rescue of organ function, patients with congenital RBC dysfunction often require repeated transfusions[7, 8]. Chronic transfusion therapy significantly decreases complications in patients with congenital hemoglobinopathies, such as sickle cell anemia or thalassemia, and serves as critical supportive therapy in patients being treated for neoplastic disease[9, 10]. However, repeat exposure to allogenically distinct RBCs

increases the risk of alloimmunization[11, 12]. Indeed, 20-50% of patients with sickle cell anemia develop alloantibodies against RBC antigens[13]. Formation of alloantibodies in these patients not only limits the therapeutic efficacy of transfused cells, but can significantly limit the availability of compatible RBCs for future transfusions. Furthermore, alloantibodies increase the probability of patients developing hemolytic transfusion reactions, one of the leading causes of transfusion related mortality[13]. As a result, a greater understanding of mechanisms of RBC alloimmunization is needed.

Although solid organs and RBCs possess the capacity to induce alloantibodies, the nature of their alloantigens fundamentally differs. For example, while solid organ rejection typically reflects alloimmunization against major histocompatibility (MHC) antigens[14], RBC alloantigens reflect a wide variety of distinct glycoproteins, such as Rh, Kell, Duffy and Kidd, which unlike MHC antigens, often display relatively RBC restricted expression with varying ability to induce alloantibody formation[13, 15]. The discovery of MHC antigens resulted from transplantation and backcrossing of inbred strains of mice[16, 17], which not only provided a unique model to study mechanisms alloimmunization to solid organs, but also enabled subsequent development of the immunomodulatory therapeutics that made transplantation a reality[6]. In contrast, no prophylactic currently exists that directly inhibits RBC alloimmunization following therapeutic RBC transfusion[13].

As examination of murine models continues to provide significant insight into solid organ transplantation[18], we likewise sought to use the most clinically relevant mouse model of transfusion in order to investigate the potential mechanisms of red cell alloimmunization. This recently described transgenic model expresses the KEL glycoprotein, one of the most common RBC alloantigens implicated in hemolytic transfusion reactions[19], under the hemoglobin promoter on murine RBCs, and is the first to closely recapitulate the essential features of RBC alloimmunization. Specifically, transfusion of KEL positive (K+) RBCs into KEL negative (K-) recipients results in robust anti-KEL antibody formation, and anti-KEL antibodies actively clear K+ RBCs and following subsequent transfusion induce rapid removal and a systemic inflammatory response[20]. In this study, we use the Kell transgenic model to demonstrate that removal of the spleen and more particularly, depletion of marginal zone B cells within the spleen, prevent IgG alloimmunization and immunological clearance of K+ RBCs in K- recipients, providing unprecedented insight into the factors responsible for RBC alloimmunization.

MATERIALS AND METHODS

Mice C57BL/6 (K-) mice were purchased from the National Cancer Institute (Frederick, MD). K+, aka KEL2 (22) transgenic mice expressing the human Kell glycoprotein were bred by the Emory Division of Animal Resources. All animals were housed in the Emory University Department of Animal Resources facilities, and all procedures and protocols were approved by the Emory University Institutional Animal Care and Use Committee.

Murine blood collection, fluorescent labeling, and transfusion K+ and K- (C57BL/6) RBCs were collected into acid-citrate-dextrose (ACD) and washed 3 times with PBS to remove residual citrate. RBCs were labeled with chloromethylbenzamido 1,1'- dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI) or 3,3'-dihexadecyloxacarbocyanine perchlorate (DiO) according to the manufacturer's instructions (Molecular Probes, Eugene OR). After labeling, the blood was mixed at a 1:1 ratio and brought to 50% hematocrit with PBS. Recipient mice were each transfused with 300μl of the blood mixture via lateral tail vein injection.

Splenectomy C57BL/6 mice were anesthetized by intraperitoneal injection of 200μl of 10mg/ml Ketamine (Hospira Inc., Lake Forest IL) and 1mg/ml Xylazine (LLOYD Inc., Shenandoah IA). Spleens were surgically removed and incisions were closed with stainless steel wound clips. Staples were removed

approximately two weeks post-surgery and mice were given at least one additional week to recover prior to transfusion.

Marginal Zone B-cell depletion To deplete marginal zone B cells, C57BL/6 were injected i.p. with 100μl of 1mg/ml rat α-mouse CD49d and 100μl of 1mg/ml rat α-mouse CD11a or isotype controls (BD Biosciences, San Jose CA), diluted in PBS to 500μl, four days prior to transfusion. To verify depletion, spleens were harvested from additional treated mice, RBCs were lysed with red cell lysing buffer (Sigma, St Louis MO), and remaining splenocytes were stained with fluorescently conjugated α-B220, α-CD21, α-CD23, α-CD3, α-CD4, α-CD8, α-CD11b, α-CD11c, α-I-A/E, α-F4/80, α-Gr-1, (BD Biosciences, San Jose CA) and analyzed by flow cytometry.

Flow Cytometry Blood was collected retro-orbitally into ACD at specific timepoints after transfusion, and the rate of clearance of K+ compared to K- RBCs was determined by flow cytometry. RBCs were also analyzed for presence of bound IgM and IgG using APC-conjugated goat anti-mouse IgM and Alexafluor®488-conjugated goat anti-mouse IgG, respectively (Jackson Immunoresearch, West Grove PA). All antibodies were used at 1:100 dilution and all samples were analyzed on a 4-color BD FACSCalibur.

Statistical Analysis Statistical significance was determined by performing a Students t-test for comparison of 2 groups or one-way analysis of variance (ANOVA) with Tukey's posttest when 3 groups were compared using Graphpad

Prism software. Error bars represent one standard deviation and differences were considered statistically significant with p-values ≤0.05.

RESULTS

In an effort to elucidate potential factors responsible for RBC alloimmunization, we recently generated a transgenic model that displays RBC specific expression of the human Kell antigen, one of the most common antigens implicated in RBC alloimmunization [21]. Importantly, using this approach, the Kell antigen resides on murine RBCs as a distinct antigen not found on WT murine RBCs, thus providing a unique, clinically relevant antigen to study the formation and potential consequences of antibody formation against RBCs following transfusion.

Although most studies examining alloimmunization and tissue rejection utilize solid organ transplantation, RBC transfusion reflects the addition of allogeneic RBC mass to a recipient's existing RBCs. As a result, traditional approaches utilized to examine discrete donor tissue at various stages following alloimmunization cannot be employed. To overcome this limitation, we labeled K+ RBCs with a distinct lipophilic dye, DiI, prior to transfusion to enable direct examination of these cells among a mixture of RBCs following transfer into Kell negative (K-) recipients. As a control, we similarly labeled K- RBCs with a fluorescently distinct lipophilic dye, DiO, and mixed K+ and K- RBCs prior to transfusion to provide an initial ratio of K+ to K- RBCs to facilitate detection of potential changes to K+ RBCs following transfusion into K- recipients (**Fig. 3.1A**). Importantly, RBCs could be detected as a discrete population of cells following

removal and each population of transfused K+ and K- RBCs could be detected as distinct fluorescent populations with the predicted antigenic properties (**Fig. 3.1B,C**), which demonstrated that unique RBC populations can be individually tracked following transfusion into recipients *in vivo*. Unlike solid organ transplantation, where thorough evaluation of the consequence of alloimmunization requires biopsy or complete removal of the donor tissue, this discrete RBC labeling allows examination of a homogenous population of donor RBCs within a mixture of RBCs following transfusion. This provides the potential to allow unique real time evaluation of the fate of transfused red blood cells using this approach.

Previous studies of our Kell transgenic model demonstrated that K+ RBCs possess the capacity to induce rapid antibody formation that engages and clears antigen positive RBCs, providing the first model that recapitulates the fundamental aspects of clinically relevant RBC alloantigens. We therefore sought to determine whether we could favorably manipulate K- recipients to prevent K+ RBC alloimmunization, similar to studies designed to inhibit allograft rejection in other settings[18, 22]. Previous studies suggest the spleen plays a central role in RBC alloimmunization and clearance[23]. Unlike solid organ transplantation, where donor antigen presenting cells may traffic to a wide variety of secondary lymphoid tissues[24], RBCs reside primarily in circulation following transfusion, where the spleen plays a central role in antibody formation against blood-borne antigens[25], providing a potentially unique and singular target to modulate

alloantibody formation against RBC alloantigens. Thus, we next directly examined the role of the spleen in alloantibody formation against the K+ RBCs. To accomplish this, we evaluated the survival of K+ RBCs following transfusion into splenectomized K- recipients. While transfusion of K+ RBCs into K- recipients with an intact spleen displayed accelerated clearance, K+ RBCs failed to display a similar level of enhanced clearance following transfusion into splenecotmized K- recipients. K+ RBC clearance in splenectomized K- recipients matched the clearance rate observed following transfusion into K+ recipients (**Fig. 3.2A,B**), strongly suggesting that the spleen may play a key role in the development of anti-RBC antibodies. However, as the spleen may also facilitate the removal of antibody-coated K+ RBCs and these cells display reduced intrinsic clearance in splenectomized recipients (**Fig. 3.2A**), we next sought to determine whether splenectomy actually resulted in reduced levels of anti-K antibody formation following K+ RBC transfusion. Examination of sera isolated from splenectomized recipients at various time points post-transfusion demonstrated that splenectomized recipients displayed a significantly reduced ability to generate IgM or IgG anti-K antibodies following transfusion (**Fig. 3.2C,D**). Consistent with this, K+ RBCs obtained from splenectomized recipients failed to display significant engagement by IgM or IgG at various points following transfusion (**Fig. 3.3A-D).** Taken together, these results strongly suggest that the increased survival of K+ RBCs in splenectomized K- recipients results from inhibition of anti-K antibody formation and that the spleen plays a central role in RBC immunization.

Although splenectomy represents a permanent intervention occasionally employed in patients with refractory autoimmune hematological conditions[26, 27], it remained possible that specific modulation of splenic constituents might also prevent or favorably alter antibody formation. Although many different leukocytes likely play a role in the generation of antibody formation against blood-borne antigens, distinct leukocyte populations that reside within the splenic marginal sinus play a unique role in detecting and responding to blood-borne pathogens [25]. Recent studies suggest that a unique population of cells, the marginal zone B cells (MZ B cells), play a critical role in the rapid IgM antibody response following engagement of blood-borne antigen[28-30]. Furthermore, as MZ B cells specifically reside within the spleen and splenectomy prevented alloimmunization, these results suggest that unique cells not found in other lymphoid compartments may be responsible for RBC alloimmunization. Equally important, recent studies demonstrate that depletion of MZ B can inhibit the initiation of antibody formation following antigenic challenge[31-33], providing a potential mechanism to directly inhibit antibody formation following RBC transfusion.

Given the rate of anti-K antibody formation in K- recipients transfused with K+ RBCs and the central role of the spleen in anti-RBC antibody formation (Fig. 2C,D), we next determined whether depletion of MZ B cells would inhibit the initial phase of antibody-induced RBC clearance. Consistent with previous

results (34), depletion of MZ B cells with anti-αL and anti-α4 integrin antibodies resulted in selective loss of MZ B cells with a reciprocal increase in the percent of FO B cells as function of total B220+ cells (**Fig. 3.4A,B**). Similarly, other prominent immunological constituents within the spleen remained largely unaffected following MZ B cell depletion (**Fig. 3.4B**). MZ B cell depletion not only significantly blunted the initial phase of accelerated RBC clearance, but allowed K+ RBCs to sustain survival for prolonged periods of time following transfusion into K- recipients (**Fig. 3.5A,B**). In contrast, K+ RBCs transfused into K- recipients treated with isotype control antibodies experienced accelerated clearance similar to previously observed following transfusion into untreated K- recipients (**Fig 3.5A,B, 3.2B**), strongly suggesting that depletion of MZ B cells may favorably modulate alloantibody formation against K+ RBCs.

To determine whether reduced clearance of K+ RBCs reflected reduced anti-K antibody formation following MZ B cell depletion, we examined sera from MZ depleted or isotype control treated K- recipients at various time points following transfusion. Depletion of MZ B cells inhibited early development of IgM anti-K antibodies and resulted in a delayed and attenuated peak of IgM, compared to isotype control treated recipients (**Fig. 3.5C**). While MZ B cells may be important in the development of functionally active anti-K IgM antibodies, IgG antibody formation typically occurs following T cell-driven follicular B cell class switching within B cell follicles[34-36]. However, K+ RBCs transfused into MZ depleted K- recipients displayed relatively unaltered clearance, suggesting that MZ depletion

may also impact IgG anti-K antibody formation. Importantly, MZ B cell depleted recipients displayed little, if any, capacity to generate significant IgG anti-K antibodies (**Fig. 3.5D**).

The ability of MZ depletion to inhibit clearance of K+ RBCs following transfusion into K- recipients, despite the formation of some IgM anti-K antibodies, suggests that the amount of anti-K antibody generated in MZ B cell recipients is below the threshold required to actively engage and induce clearance of K+ RBCs. To examine this directly, we evaluated K+ RBCs at various time points following transfusion into MZ B cell depleted or isotype control treated K- recipients for the presence of bound IgM or IgG. Although IgM anti-K could be detected in the sera 5 to 7 days following transfusion in MZ B cell depleted recipients (**Fig. 3.5C**), very little antibody could be detected on the surface K+ RBCs (**Fig. 3.6A-C**), suggesting that the antibody generated following MZ depletion failed to possess the capacity to sufficiently bind and clear K+ RBC. Similarly, virtually no IgG antibody could be detected on K+ RBCs isolated from MZ depleted recipients (**Fig. 3.6D**). These results strongly suggest that MZ B cell depletion provides an unprecedented ability to prevent the development of functionally active anti-RBC antibodies following transfusion.

The ability of MZ B cell depletion to inhibit IgG anti-K antibody formation not only provides a strategy to inhibit the reduced circulatory lifespan that K+ RBCs experience following an initial transfusion, but these results also suggest that MZ

B cell depletion prior to an initial transfusion may allow K- recipients to undergo a subsequent transfusion without an acute hemolytic transfusion reaction. To test this, MZ depleted or non-depleted K- recipients received K+ RBCs followed by a subsequent transfusion of K+ RBCs 14 days later. Subsequent transfusion of K+ RBCs into a MZ depleted recipients failed to result in significant antibody engagement (**Fig. 3.7A**). Similarly, MZ depleted recipients failed to induce accelerated clearance of K+ RBCs following the second transfusion (**Fig. 3.7B**). In contrast, K- recipients previously exposed to K+ RBCs retained the capacity to induce accelerated clearance of RBCs following additional exposure (**Fig 3.7A,B**). These results demonstrate that MZ B cell depletion provides a novel and specific therapeutic strategy to not only inhibit alloantibody-induced clearance *during* initial antigen exposure, but also enables multiple transfusions of antigen positive RBCs into antigen negative recipients without the development of a hemolytic transfusion reaction.

DISCUSSION

Despite the discovery of RBC induced alloimmunization over seventy years ago[37, 38], much remains to be learned about the immunology of RBC antibody induction and subsequent consequences. We recently generated a novel animal model using one of the most common antigens implicated in RBC alloimmunization, the Kell antigen[19, 39]. This model successfully recapitulates the essential features of clinically relevant alloimmunization in humans and provides a robust method to study this phenomenon in a highly reproducible system. [20]. In this study, we use this model to demonstrate that splenectomy or Marginal Zone B-cell depletion can inhibit anti-KEL alloantibody formation following transfusion of K+ RBCs into a K- recipient.

Blood transfusion is life saving for many individuals, including those with hemoglobinopathies such as sickle cell disease and thalassemia. These individuals require intermittent or chronic transfusion therapy to treat or prevent disease complications such as stroke, splenic sequestration, acute chest syndrome, or ineffective erythropoiesis [9, 10]. Transfusion therapy, however, is not without risk. Repeated exposure to RBC alloantigens increases the likelihood of alloantibody formation, which may not only reduce the therapeutic efficacy of a transfused unit, but also reduces the probability of finding compatible RBCs [11, 12]. Furthermore, significant alloantibodies increase the likelihood of mistransfusion or the development of a delayed type hemolytic

transfusion reaction, which in patients with sickle cell anemia can result in hyperhemolysis, which itself can be fatal[40]. Although antigen matching protocols have reduced alloantibody formation[41], significant numbers of patients (up to 40%) continue to develop RBC alloantibodies[42]. As a result, understanding the mechanisms of RBC alloimmunization and developing prophylatic interventions to prevent antibody formation will significantly aid in the successful management of patients requiring repeat RBC transfusions.

The progress made in solid organ transplantation illustrates the clinical utility of developing murine models to study alloimmunization. Indeed, murine models of transplantation not only facilitated the discovery of MHC antigens[16, 17], but provided a robust system to understand the factors and potential therapeutic targets involved in transplant rejection. Many of these studies provided the groundwork for current immunosuppressive regiments used in routine clinical practice[6]. While many studies examining MHC alloantibody formation provide significant insight into alloimmunization and immunity in general, fundamental differences between MHC and RBC alloantigens suggest that the induction and consequences of alloimmunization against these antigens likely vary[13-15]. For example, MHC antigens possess the ability to directly engage T cells and induce their activation, most RBC antigens display restricted expression and do not play a intrinsic role in the activation of adaptive immunity[43]. Thus, although murine models of solid organ transplantation may provide significant insight into general principles of alloimmunization, immune

recognition of RBC specific antigens appears to engage a distinct set of lymphocytes to initiate fundamentally different pathways of immune recognition and activation.

The ability of splenectomy or MZ B depletion to inhibit RBC antibody formation elucidates previously unrecognized features of RBC alloimmunization and illustrates fundamental differences between alloimmunization against RBC and solid organ transplantation alloantigens. Following solid organ transplantation, donor APCs possess the capacity to infiltrate donor lymphatic tissue where they activate alloreactive recipient T cells, facilitating the development of cellular and humoral immunity against donor tissue. In contrast, RBCs typically reside within the vasculature, where splenic marginal sinuses filter blood for foreign antigen. As a result, MZ B cells are uniquely positioned to respond to blood-borne antigen. Most MZ B cell responses provide rapid T cell-independent antibody responses[44, 45], which facilitate prompt neutralization of pathogens in the setting of potential infection[46]. Consistent with this, K- recipients rapidly formed antibodies following exposure to K+ RBC and depletion of MZ B cells from the spleen significantly reduced functional IgM anti-K antibody formation.

In addition to providing a rapid response to blood-borne antigen, MZ B cells can also facilitate additional antibody production in the form of affinity maturation and class switching. For example, recent studies demonstrate that

marginal zone B cells not only respond to blood-borne antigen, but also can facilitate transport of antigens to B cell follicles [29, 47, 48], providing necessary material for efficient follicle development. In addition, several studies suggest that MZ B cells can serve as potent activators of T cells, which also aid in the development of B cell follicles and class switching [28]. MZ B cells also appear to possess the capacity to directly undergo class switching [49, 50], suggesting that MZ B cells themselves may directly contribute to IgG formation following antigenic exposure. Thus, the reduced ability of MZ depleted KEL- recipients to generate IgG anti-KEL antibodies following KEL RBC transfusion suggests that MZ B cells not only play a key role in the initial immune response to blood-borne antigens, but play a central and previously unrecognized role in the general humoral immune response to the Kell antigen.

Although splenectomy prevented alloimmunization, providing a possible strategy to inhibit alloantibody formation in chronically transfused individuals, removal of the spleen represents a permanent procedure that itself can result in significant complications, such as increased rates of infection and thrombotic events[51, 52]. Furthermore, splenectomy may need to occur prior to foreign RBC antigen exposure in order to be an effective preventive measure, which simply is not feasible in most clinical settings. Selective inhibition or modulation of splenic constituents, however, provides a more specific method of inhibiting alloimmunization while reducing the deleterious consequences of removing the entire spleen. Thus, the ability of MZ B cell depletion to inhibit antibody formation

provides a specific prophylatic intervention capable of inhibiting RBC alloimmunization. Importantly, MZ B cell depletion not only reduced immune-mediated clearance of KEL RBCs following transfusion[53], but also eliminated the development of a hemolytic transfusion reaction following subsequent exposure. Thus, the ability of MZ B cell depletion to inhibit RBC alloimmunization provides the first example of a targeted prophylatic with the potential to prevent RBC alloimmunization, likely enhancing the therapeutic capacity of transfusion while increasing the availability of compatible RBCs, thus reducing the probability of a hemolytic transfusion reaction.

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FIGURE LEGENDS

Figure 3.1. Lipophilic dye labeling strategy allows for direct examination of KEL RBCs following transfusion. (A) Schematic representation of the Kell antigen on K+ RBCs. Kell antigen negative (K-) and Kell antigen positive (K+) RBCs were labeled with the lipophilic dyes, DiO and DiI, respectively followed by mixing these cells at a defined ratio prior to transfusion and examining each population for clearance and bound antibody following transfusion. (B) Gating strategy employed to examine RBCs following transfusion. (C) Incubation of RBCs post-transfusion with anti-KEL antibody followed by gating on DiO or DiI positive RBCs and examination for the Kell antigen. Results are representative of 3 independent experiments with at least 3-5 mice per group. * $p < 0.05$

Figure 3.2. Splenectomy prevents removal of and antibody formation against K+ RBCs following transfusion into K- recipients. (A) Representative dot plots of cells 14 or 21 days following transfusion into splenectomized or non-splenectomized recipients as indicated. (B) Quantitative analysis of K+ RBC clearance at the time points indicated following transfusion into non-splenectomized K- recipients, K- splenectomized recipients and K+ recipients. Error bars represent standard deviation. (C-D) Analysis of IgM anti-K (C) or IgG anti-K (D) antibodies at the time points indicated post-transfusion into non splenectomized K- (K-), splenectomized (S) K- recipients or K+ recipients as indicated. Results are representative of 2 independent experiments with at least 3 mice per group. * $p < 0.05$

Figure 3.3. Splenectomy prevents anti-K antibody engagement of K+ RBCs following transfusion into K- recipients. (A-B). Representative histograms of antibody detection of IgM antibody on DiO positive K- RBCs (grey) or DiI positive K+ RBCs (solid black line) at the times points indicated following transfusion into non splenectomized K- recipients (A) or splenectomized K- recipients (B). (C-D) Quantitative analysis of IgM (C) or IgG (D) anti-K antibody bound to K+ RBCs at the times points indicated following transfusion into non splenectomized K- recipients or splenectomized K- recipients. Results are representative of 2 independent experiments with at least 3 mice per group. * $p < 0.05$

Figure 3.4. Treatment of K- recipients with anti-αL and anti-α4 integrin antibodies results in selective removal of marginal zone B cells from the spleen. (A-) Gating strategy employed to examine B cell populations in the spleen. FO = follicular B cells, MZ = marginal zone B cell NF = non-follicular B cells. (B) Quantitative analysis of distinct leukocyte populations within the spleen following no treatment or treatment with anti-αL and anti-α4 integrins or isotype controls as indicated. Results are representative of 2 independent experiments with at least 3 mice per group. * $p < 0.05$

Figure 3.5. MZ B cell depletion prevents clearance of and inhibits antibody formation against K+ RBC following transfusion into K- recipients. (A) Representative dot plots of cells 21 days following transfusion into marginal zone

depleted or isotype control treated K- recipients as indicated. (B) Quantitative analysis of K+ RBC clearance at the time points indicated following transfusion into marginal zone depleted (MZ depleted) or isotype control treated K- recipients. Error bars represent standard deviation. (C-D) Analysis of IgM anti-K (C) or IgG anti-K (D) antibodies at the time points indicated post-transfusion into marginal zone depleted (MZD) or isotype control (IC) treated K- recipients as indicated. Results are representative of 2-3 independent experiments with at least 3 mice per group. * $p < 0.05$

Figure 3.6. MZ B cell depletion prevents anti-K antibody engagement of K+ RBCs following transfusion into K- recipients. (A-B). Representative histograms of antibody detection of IgM antibody on DiO positive K- RBCs (grey) or DiI positive K+ RBCs (solid black line) at the times points indicated following transfusion into isotype control treated K- recipients (A) or marginal zone depleted (MZ depleted) recipients (B). (C-D) Quantitative analysis of IgM (C) or IgG (D) anti-K antibody bound to K+ RBCs at the times points indicated following transfusion into isotype control treated K- recipients (IC) or marginal zone depleted (MZD) recipients. Results are representative of 2-3 independent experiments with at least 3 mice per group. * $p < 0.05$

Figure 3.7. MZ B cell depletion prevents previously transfused K- recipients from rapidly engaging and removing K+ RBCs following subsequent transfusion. (A) Representative histograms of K+ RBCs (grey) or

K- RBCs (solid black line) for IgM or IgG engagement 2 hours following a 1st or a 2nd transfusion as indicated with or without MZ B cell depletion prior to the first transfusion as indicated. (B) Representative dot plots and quantitative analysis of cells examined at the time points indicated for DiO (K-) or DiI (K+) RBCs. (C-D) Quantitative analysis of cells examined for IgM (C) or IgG (D) engagement 2 hours following a 1st or a 2nd transfusion as indicated. (E) Quantitative analysis of K+ RBC clearance at the indicated time points following a 1st or a 2nd transfusion with or without MZ B cell depletion prior to the first transfusion as indicated. Error bars represent standard deviation. Results are representative of 2 independent experiments with at least 3 mice per group. * $p < 0.05$

Figure 3.1

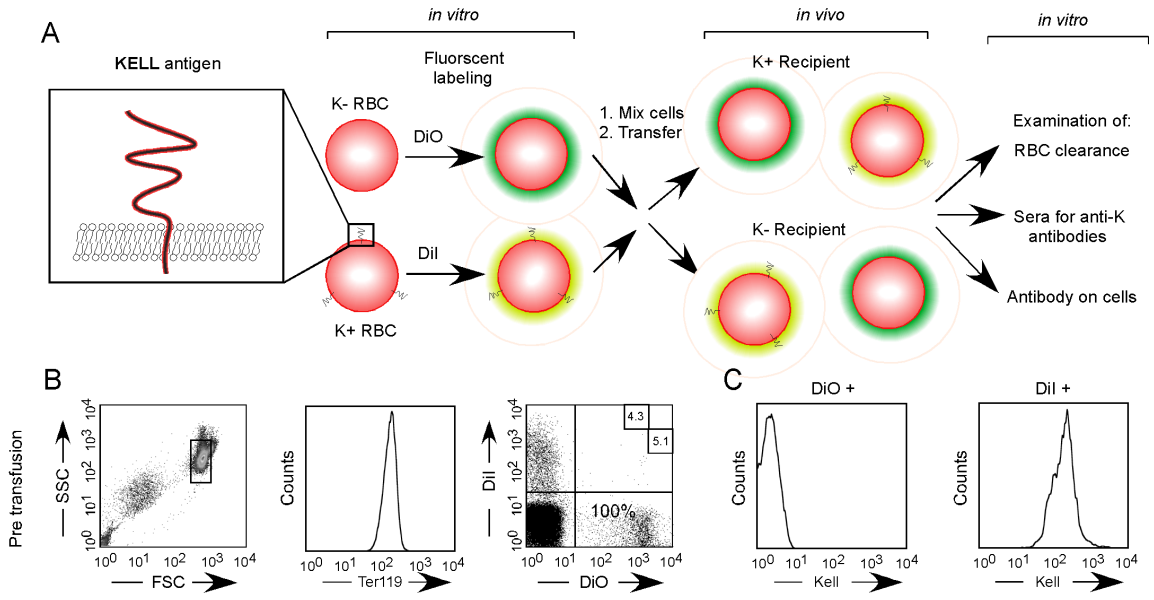

A
in vitro
in vivo
in vitro
KELL antigen
Fluorscent labeling
K- RBC
DiO
DiI
K+ RBC
1. Mix cells
2. Transfer
K+ Recipient
K- Recipient
Examination of:
RBC clearance
Sera for anti-K antibodies
Antibody on cells
B
Pre transfusion
SSC
FSC
Counts
Ter119
DiI
DiO
4.3
5.1
100%
C
DiO +
DiI +
Counts
Kell

Figure 3.2

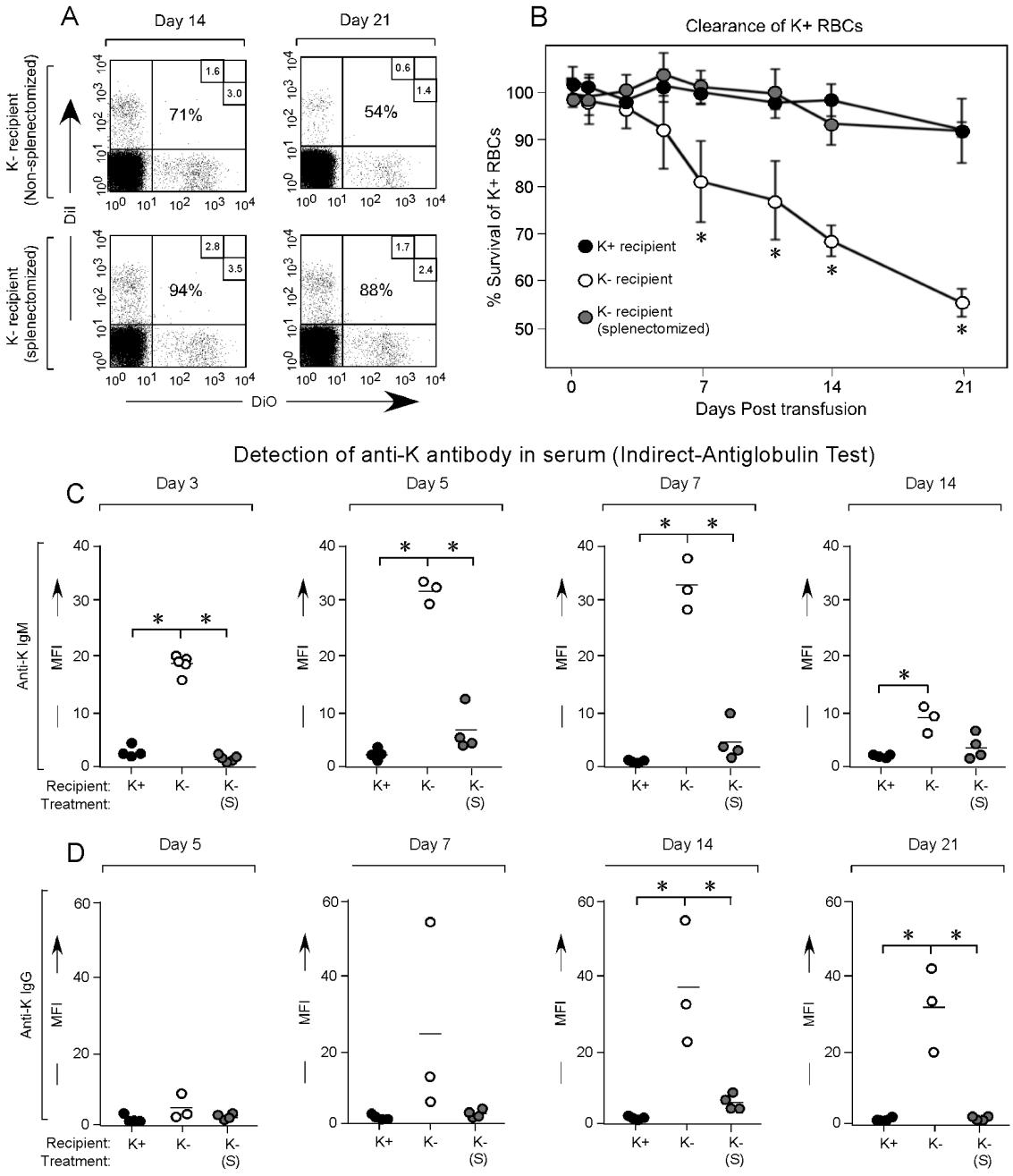

A
Day 14
Day 21
K- recipient (Non-splenectomized)
K- recipient (splenectomized)
DiI
DiO
71%
54%
94%
88%
1.6
3.0
0.6
1.4
2.8
3.5
1.7
2.4
B
Clearance of K+ RBCs
% Survival of K+ RBCs
K+ recipient
K- recipient
K- recipient (splenectomized)
Days Post transfusion
Detection of anti-K antibody in serum (Indirect-Antiglobulin Test)
C
Day 3
Day 5
Day 7
Day 14
Anti-K IgM
MFI
Recipient:
Treatment:
K+
K-
K- (S)
D
Day 5
Day 7
Day 14
Day 21
Anti-K IgG

Figure 3.3

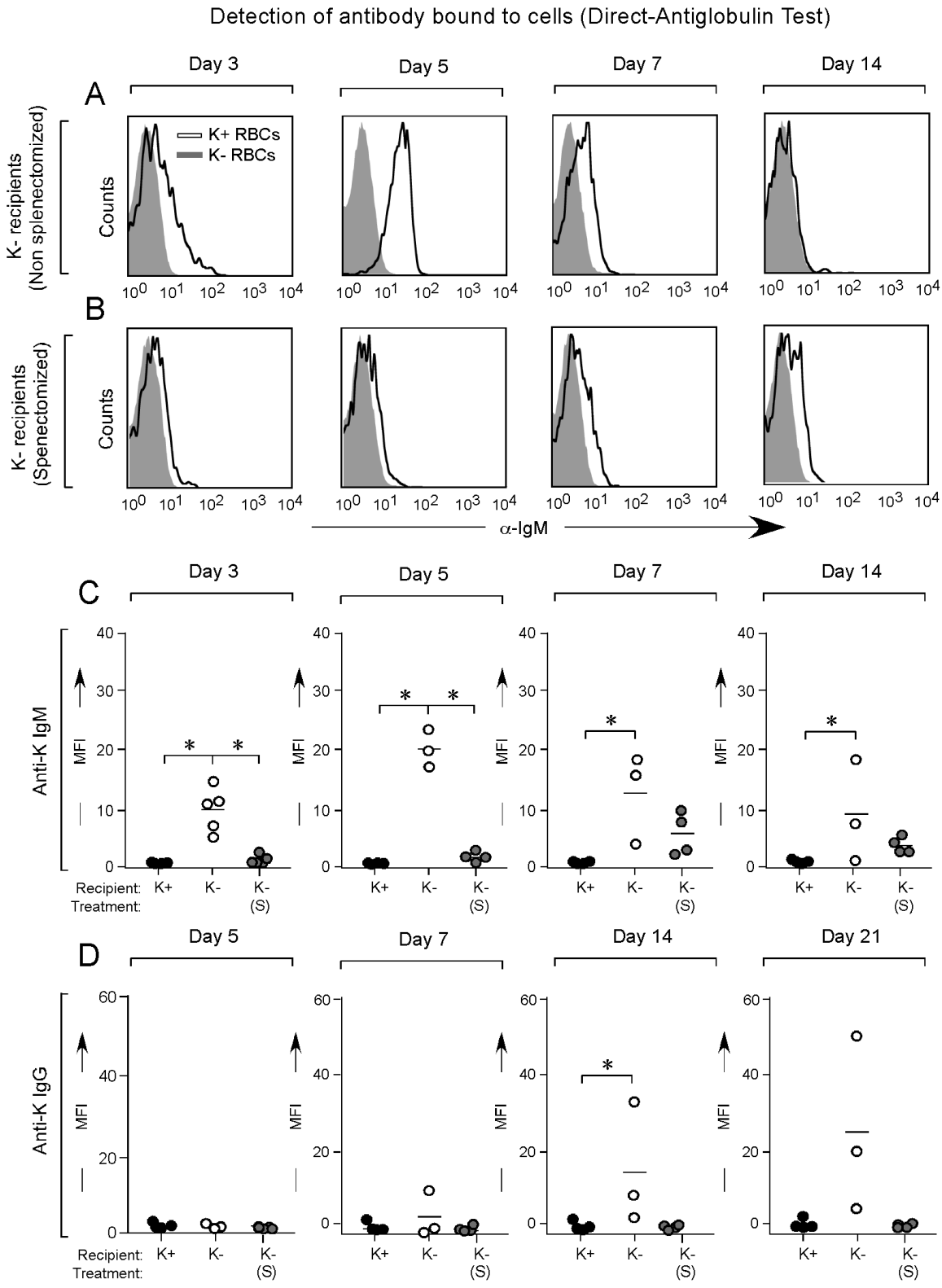

Detection of antibody bound to cells (Direct-Antiglobulin Test)
Day 3
Day 5
Day 7
Day 14
A
K- recipients (Non splenectomized)
K+ RBCs
K- RBCs
Counts
B
K- recipients (Spenectomized)
α-IgM
C
Anti-K IgM
MFI
Recipient:
Treatment:
K+
K-
K- (S)
Day 21
D
Anti-K IgG

Figure 3.4

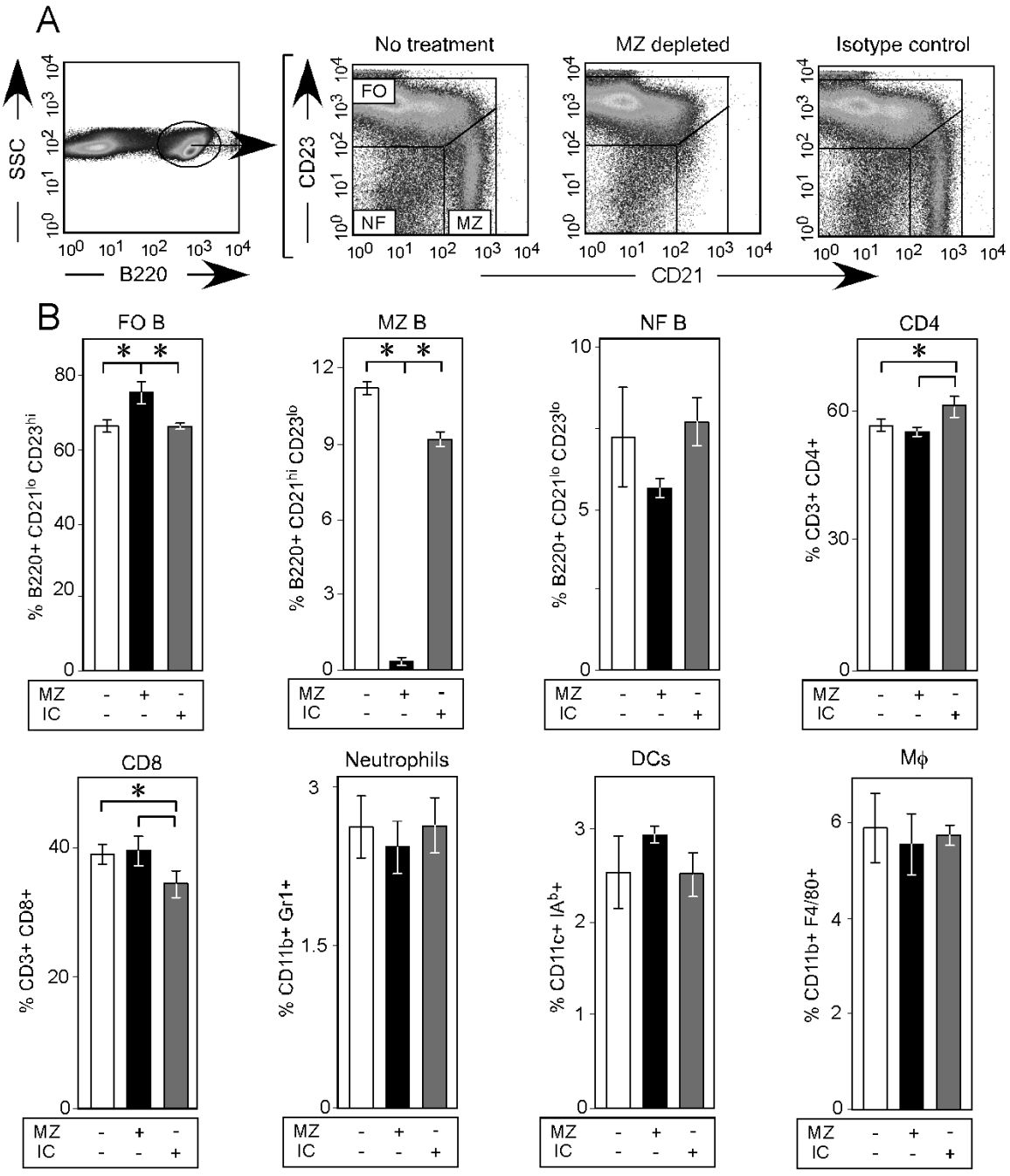

A
SSC
B220
CD23
CD21
No treatment
MZ depleted
Isotype control
FO
NF
MZ
B
FO B
MZ B
NF B
CD4
CD8
Neutrophils
DCs
Mϕ
% B220+ CD21lo CD23hi
% B220+ CD21hi CD23lo
% B220+ CD21lo CD23lo
% CD3+ CD4+
% CD3+ CD8+
% CD11b+ Gr1+
% CD11c+ IAb+
% CD11b+ F4/80+
MZ
IC

Figure 3.5

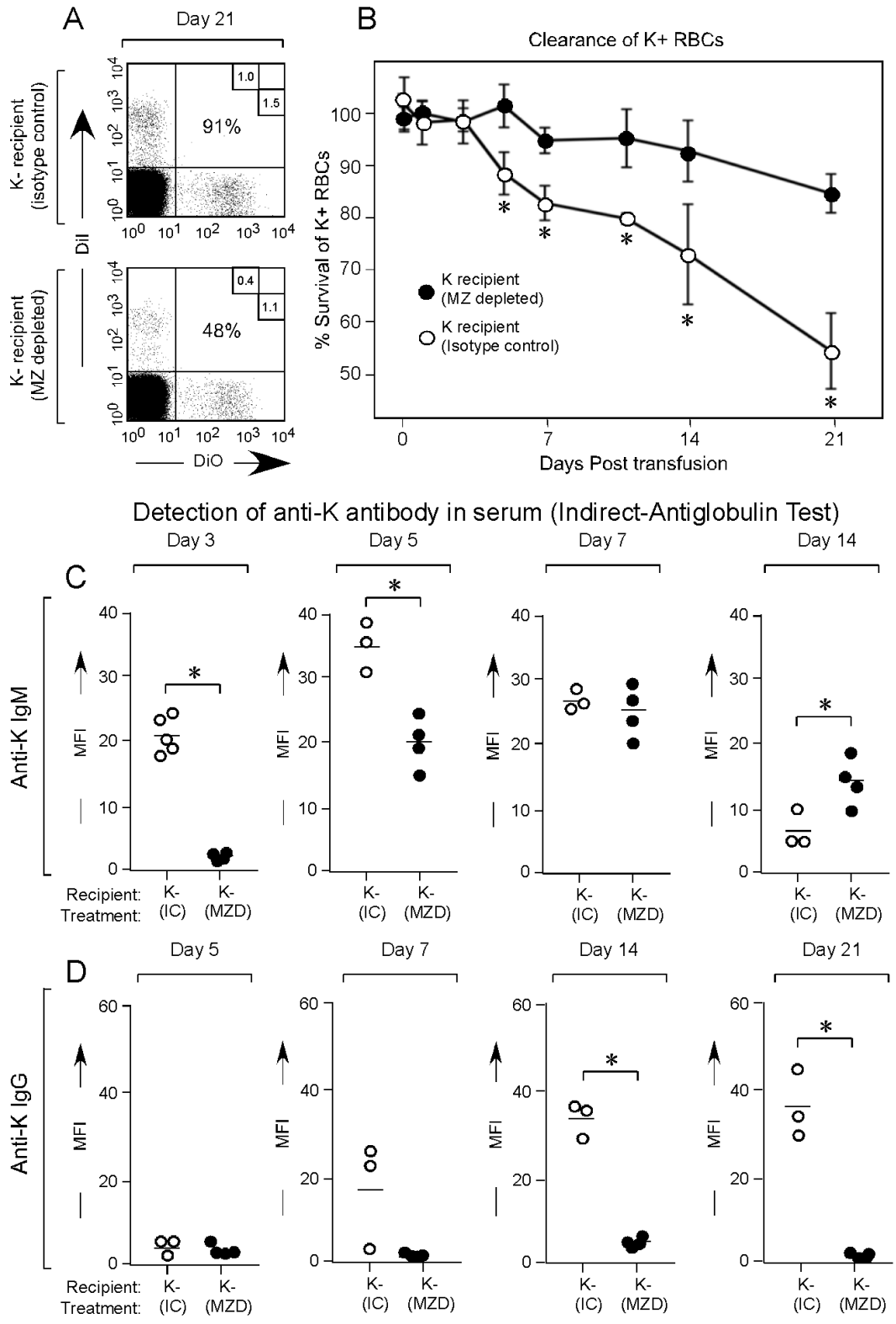

A
Day 21
K- recipient (isotype control)
91%
1.0
1.5
DiI
K- recipient (MZ depleted)
48%
0.4
1.1
DiO
B
Clearance of K+ RBCs
% Survival of K+ RBCs
K recipient (MZ depleted)
K recipient (Isotype control)
Days Post transfusion
Detection of anti-K antibody in serum (Indirect-Antiglobulin Test)
C
Day 3
Day 5
Day 7
Day 14
Anti-K IgM
MFI
Recipient:
Treatment:
K- (IC)
K- (MZD)
D
Day 5
Day 7
Day 14
Day 21
Anti-K IgG

Figure 3.6

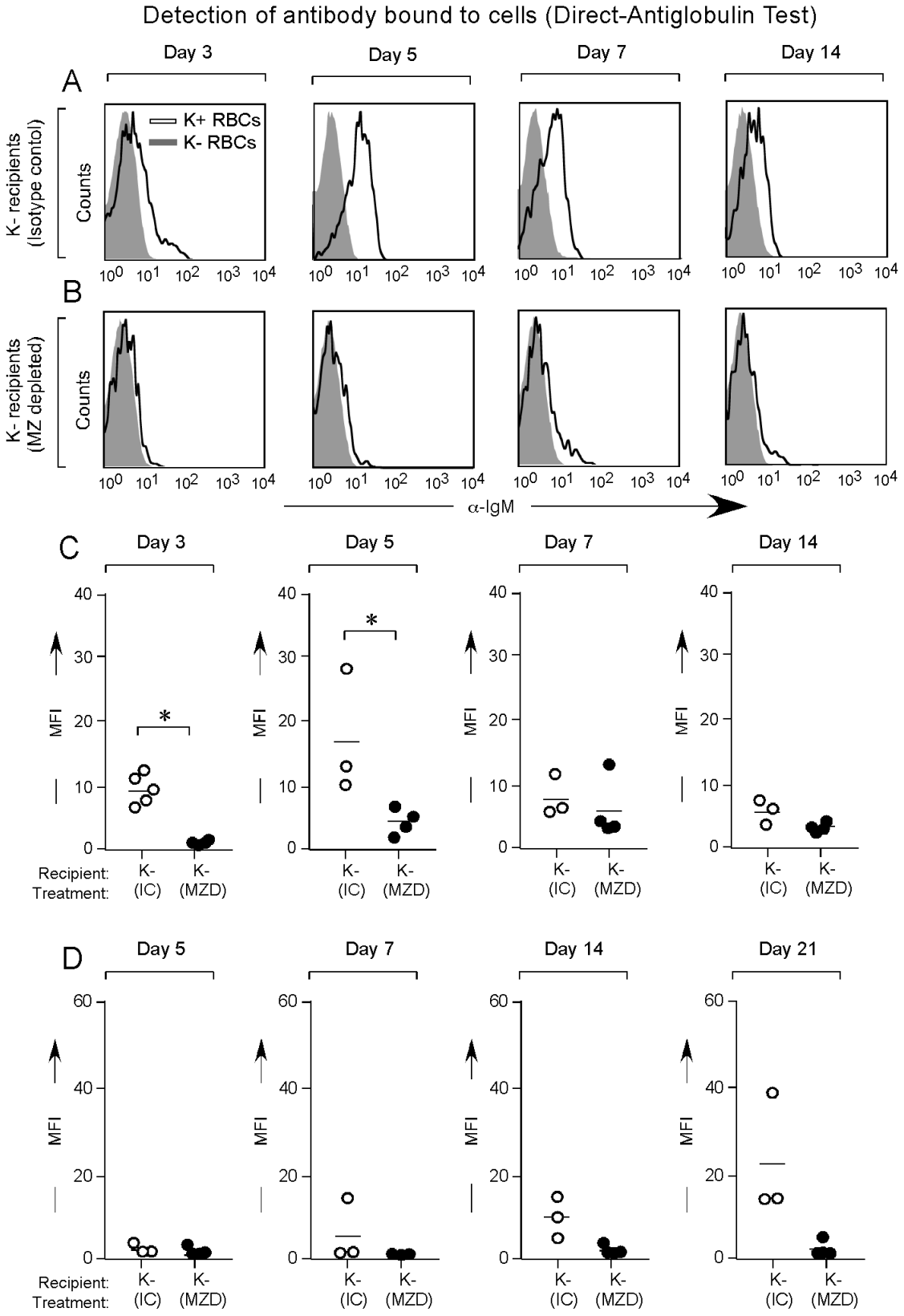
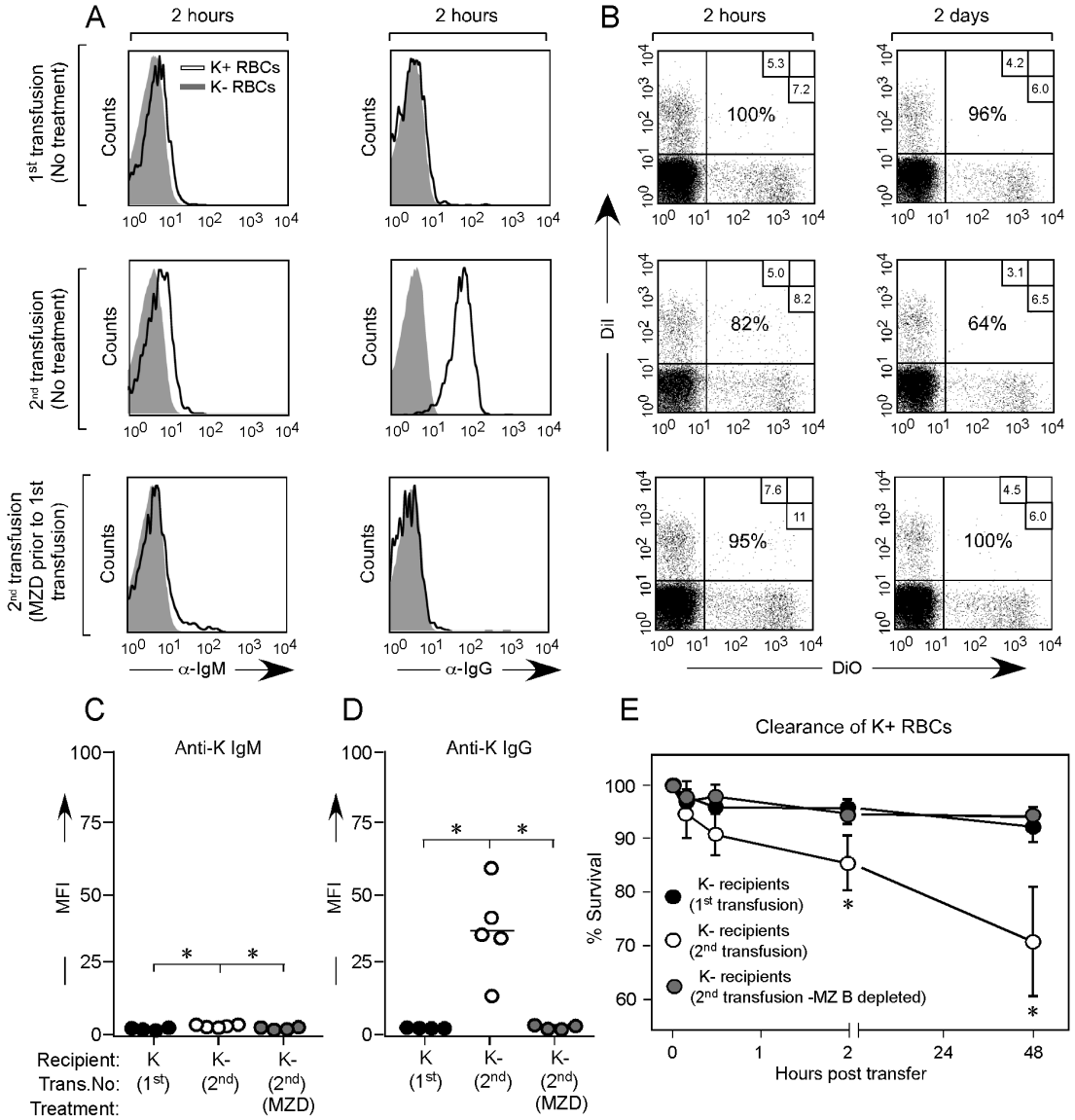

Detection of antibody bound to cells (Direct-Antiglobulin Test)
Day 3
Day 5
Day 7
Day 14
A
K- recipients (Isotype contol)
K+ RBCs
K- RBCs
Counts
B
K- recipients (MZ depleted)
α-IgM
C
MFI
Recipient:
Treatment:
K- (IC)
K- (MZD)
D
Day 21

Figure 3.7


A
2 hours
2 hours
1st transfusion (No treatment)
2nd transfusion (No treatment)
2nd transfusion (MZD prior to 1st transfusion)
K+ RBCs
K- RBCs
Counts
α-IgM
α-IgG
B
2 hours
2 days
DiI
DiO
100%
96%
82%
64%
95%
100%
C
Anti-K IgM
MFI
D
Anti-K IgG
Recipient:
Trans.No:
Treatment:
K
K- (1st)
K- (2nd)
K- (2nd) (MZD)
E
Clearance of K+ RBCs
% Survival
K- recipients (1st transfusion)
K- recipients (2nd transfusion)
K- recipients (2nd transfusion -MZ B depleted)
Hours post transfer

Chapter 4

A novel role for C3 in antibody-induced red blood cell clearance and antigen modulation

ABSTRACT

Hemolytic transfusion reactions (HTRs) due to incompatible RBC transfusions are a leading cause of transfusion-associated death. Although many transfused incompatible RBCs are cleared, some remain in circulation despite the presence of RBC specific antibodies, potentially due to “antigen-modulation.” With a goal of better understanding incompatible RBC clearance, we generated a murine model with RBC specific expression of a clinically significant human antigen (KEL2) known to be involved in antigen modulation as well as in HTRs. Wild type recipients transfused with transgenic KEL2 RBCs generated anti-KEL glycoprotein alloantibodies, which fixed complement, led to intravascular hemolysis, and resulted in decreased levels of KEL2 antigen detectable on cells remaining in circulation. Antigen modulation did not appear to solely reflect removal of RBCs with higher antigen expression, as cells continued to display antigen modulation in the absence of significant clearance. Recipients genetically lacking complement exhibited lesser degrees of incompatible RBC clearance and antigen modulation compared to wild type or FcγR KO animals, suggesting a role for complement in RBC clearance. In sum, this HTR model may serve as a platform to test strategies to down-modulate antigen and inhibit incompatible RBC clearance, thus potentially mitigating transfusion dangers.

INTRODUCTION

Complement is one of the major effector pathways by which antibodies destroy cellular targets to which they bind[1-4]. C3 plays a pivotal role in this process by serving both as a direct opsonin after attaching to surfaces in the form of C3b and also by leading to downstream assembly of the membrane attack complex[5-7]. In addition to fixation of C3, antibodies can opsonize cellular targets through ligation of Fc gamma receptors (FcγRs)[8, 9]. Although straightforward in concept, the destruction of targets by antigen bound antibodies is a dynamic process, with multiple regulatory components[10, 11]. For example, self-tissues have several pathways that actively inhibit complement activation. Moreover, once deposited, C3b is broken down into iC3b, C3d and C3dg[12]. In this way, self-tissues have evolved methods to avoid destruction by binding of self-antibodies.

In addition to regulating the effector function of bound antibodies, the targets of antibody binding can undergo compensatory changes. Antigen-modulation is a process by which target cells alter the antigens being recognized by the antibody in question. Antigen-modulation occurs in multiple settings with different target tissues and antigens, including: nicotinic cholinergic receptors in Myasthenia Gravis, desmogleins in Pemphigus Vulgaris, glycoproteins on platelets, and HLA on transplanted tissues[13]. The phenomenology of antigen-modulation in humans has been repeatedly described in the context of antibodies binding red blood cells (RBCs) [14-20]. This outcome has been termed “depressed antigen”, “antigen suppression”, “weakened antigenicity” and “antigen

loss" and has been observed for multiple blood group antigens, including Kell, RhD, RhC, Rhe, Jka, Jkb, Gerbich, LW, AnWJ, and Cromer. Among these, antigen-modulation has been described most frequently for antigens in the Kell system. Nevertheless, despite the multiple settings in which antigen-modulation occurs and its frequency in RBC biology, relatively little is known about its mechanistic underpinnings.

While immune mediated destruction of RBCs is often the result of auto or alloantibody binding, it is not the inevitable outcome. In some cases, transfusion of a unit of RBCs against which a recipient has an alloantibody (*i.e.* an "incompatible" unit) causes no clinical symptoms, with the transfused RBCs remaining in circulation and the hematocrit increasing appropriately [21, 22]. In other cases, all of the incompatible RBCs clear rapidly in a hemolytic transfusion reaction (HTR), potentially resulting in coagulopathy, renal failure, and death. In fact, HTRs are a leading cause of transfusion-associated death. The mechanism(s) by which anti-RBC antibodies have such different effects remain poorly understood; however, antigen-modulation has been described in some RBCs that escape destruction from allo or autoantibodies.

To investigate antigen-modulation in a reductionist setting, we have recently described a transgenic mouse model with expression of the human Kell glycoprotein (KEL2) specifically on RBCs. The RBCs from KEL2 mice have a normal circulatory lifespan, and are recognized by antibodies that bind to the main significant antigens in the Kell system, in particular KEL2, Kp^b, and Js^b [23]. We have recently reported KEL2 RBCs transfused into wild-type recipients

induce an anti-KEL glycoprotein response[24]. Herein, we report that anti-KEL immunoglobulins induce antigen-modulation on incompatible KEL2 RBCs, with C3 playing a role not only in this antigen modulation but also in cellular clearance. FcγRs likewise contribute to RBC clearance in this Kell model, but are not required for antigen-modulation. In aggregate, these studies provide the first report of complement-associated antigen-modulation using an authentic human RBC antigen system, for which HTRs and antigen-modulation have also been observed in humans.

.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). *KEL2* transgenic mice expressing the human KEL glycoprotein were generated by our laboratory. Donor KEL2 RBCs have approximately 2000 copies of the KEL2 antigen on their cell surface and have previously been published as "KEL2A[23]." C3 KO and common gamma chain (FcγR) KO (Fcer1g) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and Taconic Farms (Hudson, NY), respectively. All mice used in these studies are on a C57BL/6 background, thus avoiding any potential MHC mismatch. All animals were housed in the Emory University Department of Animal Resources facilities, and all procedures and protocols were approved by the Emory University Institutional Care and Use Committee

Antibodies and passive immunization

Antiserum against the KEL glycoprotein was generated by transfusing KEL2 RBCs into recipients pre-treated with an intraperitoneal injection of 100μg poly (I:C) (Amersham/GE Healthcare, NY) a total of 3 times, separated by two weeks. Pooled sera collected 2 weeks after the final transfusion was tested for KEL binding ability by flow crossmatch using KEL2 or control C57BL/6 RBCs as targets, using APC-conjugated goat anti-mouse IgG (BD Biosciences) or HRP conjugated goat anti-mouse IgM or IgG subtypes (Bethyl Laboratories,

Montgomery, TX), plus anti-HRP Cy5.5 (Jackson Immunoresearch Laboratories, West Grove, PA). Because the entire human KEL glycoprotein is present on donor RBCs but absent on recipient RBCs, the antibody response is “anti-KEL glycoprotein”, referred to herein as anti-KEL; however, this is not to be confused with antibodies specific for human polymorphisms (e.g. anti-KEL1 or anti-KEL2). In passive immunization experiments, recipient mice were given 25μl anti-KEL antiserum intravenously 2 hours prior to transfusion.

Murine blood collection, fluorescent labeling, and transfusion

Donor KEL2 or wild type C57BL/6 RBCs were collected into acid citrate dextrose (ACD) and washed three times to remove residual citrate. Prior to transfusion, RBCs were labeled with chloromethylbenzamido 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (CM-DiI) or 3,3’-dihexadecyloxacarbocyanine perchlorate (DiO) according to the manufacturer’s instructions (Molecular Probes, Eugene OR) and as previously described[25]. After labeling, cells were washed at least three times to remove any unbound dye. Experimental and control RBCs were mixed at a 1:1 ratio and recipient mice were transfused via lateral tail vein with 50-75μl (equivalent of one human unit) of each type of blood. Survival of the transfused RBCs was determined by comparing the ratio of circulating KEL2 RBCs to control RBCs in recipients at select time points post-transfusion; in some experiments, post-transfusion blood smears were Wright-Giemsa stained and analyzed.

Cytokine and Urine Analysis

Serum was evaluated post-transfusion for Interleukin (IL)-6, keratinocyte-derived chemokine (KC), monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1β , using a Cytometric Bead Array Mouse Flex Kit (BD Biosciences, San Diego, CA); heme was measured by cyanomethemoglobin assay. Urine was evaluated post-transfusion, with absorbance measured at OD 414 nm.

Flow cytometry

Transfused RBCs were analyzed for presence of bound anti-KEL antibody by performing a direct antiglobulin test (DAT), using Alexafluor®488-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove PA) or goat anti-mouse immunoglobulin conjugated to allophycocyanin (BD Biosciences PharMingen, La Jolla, CA). Antigen levels were determined by staining transfused RBCs with anti-KEL antisera, followed by the same secondary antibody. Transfused RBCs were analyzed for binding of all forms of C3 or active forms of C3 using biotinylated rat anti-Mouse Complement Component C3 (Clone RmC11H9) or mouse anti-Human/Mouse C3/C3b/iC3b (Clone: 10C7) (Cedarlane, Ontario, Canada), respectively, followed by Streptavidin conjugated to allophycocyanin secondary (BD Biosciences PharMingen, La Jolla, CA). All antibodies were used at a 1:100 dilution and samples were analyzed on a 4-color BD FACSCalibur.

Statistical analysis

All statistical analysis was performed using Graphpad Prism software (San Diego, CA). A student's t-test was used to determine significant differences between groups. Error bars represent one standard deviation and significance was determined by a p-value of ≤0.05.

RESULTS

Transfusion of KEL2 RBCs induces anti-KEL glycoprotein alloantibodies

To determine baseline immune responses to KEL2 RBCs, wild type C57BL/6 (B6) mice were transfused with the volume adjusted equivalent of 1 human unit of KEL2 RBCs. Immune responses were tracked serially post-transfusion by flow cytometry, using KEL2 or B6 RBCs as targets (**Figure 4.1A**). B6 recipients of KEL2 RBCs generated anti-KEL glycoprotein IgG, which was detectable in the serum approximately 5-7 days post transfusion, and increased over time. **Figure 4.1B**). This was not a non-specific antibody, as it bound to KEL2 but not B6 RBCs; furthermore, no anti-KEL was detected in syngeneic recipients following KEL2 RBC transfusion.

Although nearly 100% of transfused B6 mice generated anti-KEL glycoprotein antibodies following a single transfusion of KEL2 RBCs, the antibody levels varied between recipients. To study the mechanisms of RBC clearance in animals with uniform levels and composition of anti-KEL, we therefore switched to a passive immunization model. To generate antisera for this model, B6 mice were transfused three times with KEL2 RBCs and antisera was pooled 2 weeks after the final transfusion. The IgM and IgG subtype composition of this antisera was determined by flow cytometric crossmatch (**Figure 4.1C**). This antisera was then injected i.v. into recipient mice to allow passive immunization with a defined titer and composition of anti-KEL.

Passive immunization of polyclonal anti-KEL results in incompatible RBC clearance

Clearance of the KEL2 *RBCs* in animals passively immunized with polyclonal anti-KEL was tracked by evaluating the ratio of DiI positive KEL2 RBCs to DiO positive B6 RBCs (**Figure 4.2A** shows gating strategy). By analyzing KEL2 RBC survival as a function of B6 RBC survival, we were able to control for factors such as injection volume, sera collection over time, and non-antigen specific clearance due to normal cell turnover or damage during collection or labeling. A titration of anti-KEL showed essentially no clearance of KEL2 RBCs at very low doses, with maximal clearance noted using 25 μL (**Figure 4.2B**). No additional clearance was observed with higher amounts of antisera, and no KEL2 RBC clearance was observed in the saline control arms (**Figure 4.2C**). All subsequent studies were performed using a 25 μl dose of anti-KEL, with analyses limited to the first 24-48 hours post-transfusion to avoid the confounder of the recipients' own anti-KEL responses.

Following incompatible KEL2 RBC transfusion, serum was evaluated for cytokines, serum and urine were evaluated for free heme, and blood smears were generated. Elevations in KC, MCP-1, MIP-1β, and TNF-α were observed 2 hours after the transfusion of KEL2 RBCs in immunized but not non-immunized recipients (**Figure 4.2D**). Additionally, free heme was noted in the serum visually as well as by cyanomethemoglobin assay (**Figure 4.2E**), and heme was noted in the urine visually as well as by OD414 absorbance (**Figure 4.2F**). Analysis of the Wright Giemsa stained blood smears under a brightfield microscope showed no

obvious increase in spherocytes (**Figure 4.2G**), though our detection abilities were limited given transfused blood volumes. Taken together, these data suggest that intravascular hemolysis is playing a significant role in the early clearance of incompatible KEL2 RBCs.

C3 binds to transfused KEL2 RBCs in immunized recipients and is rapidly inactivated

C3 deposition is a key component both in antibody induced opsonization (through C3 receptors) and through generation of downstream complement components that lead to assembly of the membrane attack complex (MAC). To test the extent of C3 deposition in response to anti-KEL binding of KEL2 RBCs, the DAT approach above was modified by using anti-C3. Utilizing an antibody that recognizes all forms of C3, we observed substantial reactivity with anti-C3 on KEL2 but not B6 RBCs after an incompatible transfusion (**Figure 4.3A**, representative histograms are provided below graph). C3 deposition on KEL2 RBCs was detected as early as 10 min post-transfusion, and then progressively diminished over time. This bound C3 was not an artifact of the passive immunization model, as C3 was also observed during primary active immunization (data not shown). To monitor C3b degradation, DATs were performed with an antibody that recognizes C3b and iC3b but not C3g or C3dg. C3/C3b/iC3b was detectable at early time points; however, unlike total C3 staining that was detected out to 24 hours post-transfusion, C3/C3b/iC3b staining became essentially undetectable by 2 hours (**Figure 4.3B**).

Both C3 and FcγRs participate in clearance of KEL2 RBCs by anti-KEL

To assess the functional role that C3 plays in clearance of KEL2 RBCs by anti-KEL, we utilized recipients with a targeted deletion of the C3 gene (C3 KO). C3 KO recipients consistently had lower levels of RBC clearance than did wild-type B6 mice (**Figure 4.4A**). However, clearance of RBCs was not eliminated in the C3 KO mice. To assess the role of FcγRs in KEL2 RBC clearance, analogous studies were performed in mice with a targeted deletion of the common gamma chain for FcγRs (γ-chain KO mice). γ-chain KO mice lack functional FcγRI, FcγRIII, and FcγRIV. Clearance of KEL2 RBCs by anti-KEL was also decreased in γ-chain KO mice compared to wild type B6 (**Figure 4.4A**). Our interpretation of these data is that C3, FcγRI, FcγRIII, and FcγRIV are involved but not solely responsible for clearance of incompatible KEL2 RBCs.

Anti-KEL induces antigen modulation of KEL2 in surviving RBCs by a mechanism involving C3

To test the extent to which IgG remained bound to KEL2 RBCs that survived incompatible transfusion, DAT analysis was performed using an anti-IgG reagent. Anti-IgG was readily detected after gating on KEL2 RBCs but not B6 RBCs (**Figure 4.5A** shows representative histograms). This signal was specific for anti-KEL, as recipients transfused with saline instead of polyclonal anti-sera had essentially negative DATs, and B6 DiO labeled RBCs also had essentially

negative DATs. The intensity of anti-IgG staining progressively decreased over 24 hours in wild type B6 mice, with a similar decline observed in γ-chain KO mice. In contrast, a lesser decrease in detectable IgG with slower kinetics was observed in C3 KO mice. (combined data in **Figure 4.5B**).

To test the hypothesis that antigen-modulation may be induced by incompatible transfusion, we analyzed levels of KEL glycoprotein on circulating KEL2 RBCs by staining with the same polyclonal anti-KEL utilized for passive immunization. Similar to what was observed with respect to bound anti-KEL IgG in the DATs, the level of detectable KEL glycoprotein also decreased over time in both wild type B6 as well as γ-chain KO recipients (**Figure 4.6A** shows representative histograms, combined data shown in graph form in **Figure 4.6B**). In contrast to what was observed in wild type or γ-chain KO mice animals, C3 KO recipients had fairly stable levels of KEL2 antigen detected out to 2 hours post-transfusion. However, a decrease in detectable KEL2 antigen was observed in C3 KO mice, albeit to a lesser degree and with slower kinetics than in wild type animals. Taken together, these data suggest that antigen-modulation occurs on KEL2 RBCs that survive incompatible transfusion, and that C3 is likely involved in this process.

Selective destruction of RBCs with the highest level of KEL glycoprotein may occur at early but not later post-transfusion time points

A simple explanation for the observed changes in KEL2 antigen level following incompatible transfusion is that the cells with the highest levels of KEL2 antigen

are the most susceptible to complement-mediated lysis. To test this hypothesis, we compared the percent of incompatible KEL2 RBCs that cleared over discrete time intervals post-transfusion to the percent of cells experiencing “antigen modulation” over the same period of time. In a representative experiment, 41.5% of KEL2 RBC transfused into a wild type mouse in the presence of passively administered anti-KEL cleared in the initial 10 minutes after transfusion, 12.8% cleared between 10 minutes and 1 hour after transfusion, and 17% cleared between 1 and 24 hours after transfusion (**Figure 4.7A).** In contrast, there was a 54.8% change in KEL2 antigen detected at 10 minutes compared to pre-transfusion, a 48.5% change in KEL2 antigen detected at 1 hour compared to 10 minutes, and a 65.4% change in KEL2 antigen detected at 24 hours compared to 1 hour post-transfusion (**Figure 4.7B)**. Juxtaposition of graphs comparing clearance of circulating cells to a change in KEL2 antigen detectability (**Figure 4.7C**) suggests that although “antigen modulation” may simply reflect removal of the KEL2 RBCs with the highest level of expression, the percent of cells undergoing clearance at the later time intervals is significantly outweighed by the percent of cells experiencing antigen modulation, strongly suggesting that selective destruction of the highest expressing KEL2 RBCs does not account for the antigen modulation observed at these later time points post-transfusion.

DISCUSSION

These studies describe a murine model in which polyclonal anti-KEL immunoglobulins are generated through transfusion[24] and are used to examine clearance following passive immunization. The RBC biology of incompatible transfusion in this model is multifaceted, with rapid intravascular clearance of a subset of incompatible RBCs, yet persistent circulation and apparent antigen-modulation of other circulating transgenic RBCs. Similar findings have also been described in humans, with hemolysis of a subset of Kell RBCs yet antigen modulation of surviving Kell RBCs [14-20, 26]. Thus, the RBC clearance, RBC survival, and antigen-modulation observed in our murine studies parallel what is known to occur in humans.

The current model is one of the first described in which C3 is involved not only in antibody induced incompatible RBC clearance, but also in apparent antigen-modulation. C3 KO recipients not only have blunted RBC clearance rates but also have lesser degrees of antigen modulation than wild type recipients. It has been shown that C3 can be fixed after antibody binding to an extent that the bound C3 obfuscates binding of the detection immunoglobulin, and the most simplistic explanation of our findings is that C3 is merely masking the detectable antigen. However, a careful analysis of the kinetics seems to rule out this possibility. In particular, the C3 specific DAT detects peak C3 fixation by 10 minutes post-transfusion and yet antigen-modulation is ongoing during a post-transfusion interval (2 hours to 24 hours) that all detectable forms of C3 are decreasing. Thus, engagement of one of several distinct complement receptor

pathways may be important in complement-mediated antigen modulation. Ongoing studies are exploring these possibilities.

It is theoretically possible that antigen-modulation following incompatible transfusion simply reflects selective removal of antigen high cells within the transgenic antigen positive population. Consistent with this, the percentage of cells undergoing clearance within the first 10 minutes post-transfusion parallel the percent experiencing antigen modulation. However, close analysis of antigen modulation and clearance over subsequent time intervals demonstrates that a much higher percentage of cells experience antigen modulation than undergo clearance, strongly suggesting, at least for these later time intervals, that antigen-modulation does not solely reflect preferential clearance of cells with higher levels of antigen. Furthermore, the percentage of cells experiencing antigen-modulation is significantly attenuated in C3 KO recipients, despite clearance of over half the cells by 24 hours post-transfusion. In addition, antigen-modulation in this incompatible RBC setting does not result in the presence of detectable spherocytes by blood smear or to obvious changes in RBC size by flow cytometry. Taken together, these data support a process of antigen-modulation involving complement, and a process that is not solely an artifact of complement lysis-dependent selection of clearance of RBCs with the highest levels of KEL2 expression.

Analogous to the initiation of distinct effector pathways following antibody engagement, the results of the present study suggest that similarly unique pathways likewise evolved to regulate antibody-antigen interactions. For

example, recent studies have demonstrated that antigen-modulation also occurs following antibody engagement of membrane bound hen egg lysozyme (mHEL) on transgenic RBCs following transfusion[25, 27]. However, in contrast to the present findings, antibody-induced modulation of mHEL not only occurs in the absence of complement, but also requires Fc gamma receptors to be initiated. Although Fc receptors appear to play some role in antigen modulation following anti-KEL2 antibody engagement, C3 appears to play a primary role in antigen modulation in the present model, providing to unique, complement dependent mechanism whereby cells experience significant alterations in antigen following antibody engagement. It remains possible that the primary antigen-modulation pathway engaged following antibody binding in various settings reflects the principle effector pathway utilized for clearance, as complement also appears to play a more significant role in anti-KEL2-mediated clearance. Regardless, these results suggest that different antibody effector mechanisms likely possess distinct regulatory pathways that may ultimately result in a similar outcome of antigen-modulation following antibody engagement.

Although the vast majority of antibody contained in the polyclonal preparation utilized herein for passive transfer was IgG, a small amount of IgM was also present. Ongoing experiments are evaluating the ability of different class switched monoclonal antibodies to bind to KEL2 RBCs, fix C3, and lead to clearance. It must also be stated that the polyclonal anti-KEL preparation utilized in these studies is against the entire KEL glycoprotein and not merely against the KEL1 or KEL2 epitopes, and it is known that antibody clustering impacts C3

binding efficiency. Thus, although this is a model of HTRs, the antigen/antibody characteristics and interactions are not exactly like that observed in the human setting involving anti-KEL1 or anti-KEL2 alloantibodies[28, 29]. Efforts are ongoing to generate transgenic founder animals with different levels of KEL expression, which will serve to further investigate the importance of antibody clustering in C3 binding and incompatible RBC clearance.

In addition to its destructive role, C3 has also been reported to be protective towards cells[30, 31]. The cells that remain in circulation beyond 10 minutes in our model have significant levels of bound “inactive” complement, staining positive for C3 but not the active C3, C3b or iC3b. Thus one could consider a scenario in which active forms of complement play a destructive role with regards to incompatible KEL2 RBC clearance and yet inactive forms play a protective role. Ongoing studies are investigating whether complement binding or other methods of in vitro antigen modulation may allow for safer transfusion of RBCs in certain situations involving highly alloimmunized recipients.

Mechanistic analysis of clearance pathways in the current system results in a complex landscape with multiple participants. Deletion of the γ-chain (which results in lack of functional FcγRI, FcγRIII, or FcγRIV) or deletion of C3 decreases but does not eliminate clearance. This suggests that both FcγRs and C3 are involved in RBC clearance yet other pathways potentially exist. Because the γ-chain KO mice have intact FcγRIIb signaling, it also remains possible that FcγRIIb is responsible for the remaining clearance in γ -chain KO mice; however, FcγRIIb has generally been observed to function as an inhibitory pathway and is

not widely known to participate in active clearance[32, 33]. In addition, we have recently reported that some antibody induced RBC clearance pathways function through mechanisms involving neither C3 nor Fcγ Rs[34]. Thus, the residual clearance in this system may represent such alternate clearance biology.

The ability to prevent C3 mediated destruction of both allogeneic and self RBCs may be clinically beneficial in certain situations. For example, at times it is impossible to locate compatible RBCs for transfusion in highly alloimmunized individuals and the ability to "mask" incompatible antigens, either through inactive complement or other mechanisms, may allow for safer transfusion. The use of complement receptor inhibitors has in fact been proposed in such situations, though targeted therapy of the RBCs themselves may be an even more desirable strategy[35, 36]. Recently, the use of C1 esterase inhibitor concentrates (C1-INH) has been described in a refractory case of C3 mediated autoimmune hemolytic anemia, with an increase in hemoglobin and a decrease in lactate dehydrogenase levels suggesting a therapeutic benefit[37].

In sum, our murine model with RBC specific expression of a clinically significant human RBC antigen is one of the first to be described in which C3 plays a role in clearance as well as probable antigen- modulation. This model begins to add to the understanding of antigen- modulation, a phenomenon now reported multiple times in both humans and animals, in many different settings. In addition to providing evidence that C3 is likely playing a role in RBC antigen-modulation, our model may also serve as a platform for the future development

of strategies to minimize the dangers of RBC incompatibility through intentional antigen- modulation or through alteration of clearance pathways involving C3.

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FIGURE LEGENDS

Figure 4.1: Anti-KEL glycoprotein antibodies are generated in response to transfused KEL2 RBCs. Wild type and KEL2 recipient mice were transfused with KEL2 RBCs and anti-KEL glycoprotein levels were evaluated by flow cytometry post-transfusion. **A)** Flow cytometric crossmatch method for analysis of serum antibody levels. **B)** Serum IgG levels were determined by flow cytometric crossmatch in wild type (light circles) and KEL2 (dark squares) recipients at specific timepoints after transfusion. **C)** Composition of anti-sera passively infused in subsequent experiments. These data are representative of 3 independent experiments, with 3-5 animals/group/experiment; error bars indicate standard deviation and * represents statistical significance. * $p<0.05$.

Figure 4.2: Anti-KEL antibodies induce clearance of KEL2 RBCs. Naïve wild type recipients were passively immunized with PBS or titrated amounts of anti-KEL antiserum and subsequently transfused with DiI labeled KEL2 and DiO labeled wild type RBCs. **A)** Gating strategy of DiI positive KEL2 or DiO positive B6 RBCs. **B and C)** Clearance was determined by calculating the ratio of DiI labeled KEL2 to DiO labeled wild type RBCs after passive antibody transfer. **D**) Serum cytokines 2 hours post-transfusion. **E)** Heme in serum and in **F)** urine post-transfusion. **G**) Wright Giemsa stained blood smears. These data are representative of 2 independent experiments, with 3-5 animals/group/experiment;

error bars indicate standard deviation and * represents statistical significance. * $p<0.05$.

Figure 4.3: C3 is rapidly inactivated after binding to transfused incompatible KEL RBCs. A) Total C3 bound to circulating KEL2 RBCs in animals passively transferred with polyclonal anti-KEL (black bars, solid histograms) or saline (open bars, shaded histograms); representative plots are shown. **B)** Active forms of C3 (C3/C3b/iC3b) bound to circulating KEL2 RBCs in animals passively transferred with polyclonal anti-KEL (black bars, solid histograms) or saline (open bars, shaded histograms). These data are representative of 3 independent experiments, with 3-5 animals/group/experiment.; * $p<0.05$.

Figure 4.4: Clearance of transfused KEL2 RBCs occurs in part through FcγR-mediated or C3-mediated pathways. Naïve wild type, C3KO, or FcγR KO recipients were passively immunized with PBS or 25ul of anti-KEL antiserum and subsequently transfused with DiI labeled KEL2 and DiO labeled wild type RBCs. **A)** Clearance curves of DiI positive KEL2 RBCs remaining in circulation post-transfusion in wild type or C3KO recipients passively transferred with polyclonal anti-KEL (dashed line) or saline (solid line). **B)** Clearance curves of DiI positive KEL2 RBCs remaining in circulation post-transfusion in wild type or FcγR KO animals passively transferred with polyclonal anti-KEL (dashed line) or saline

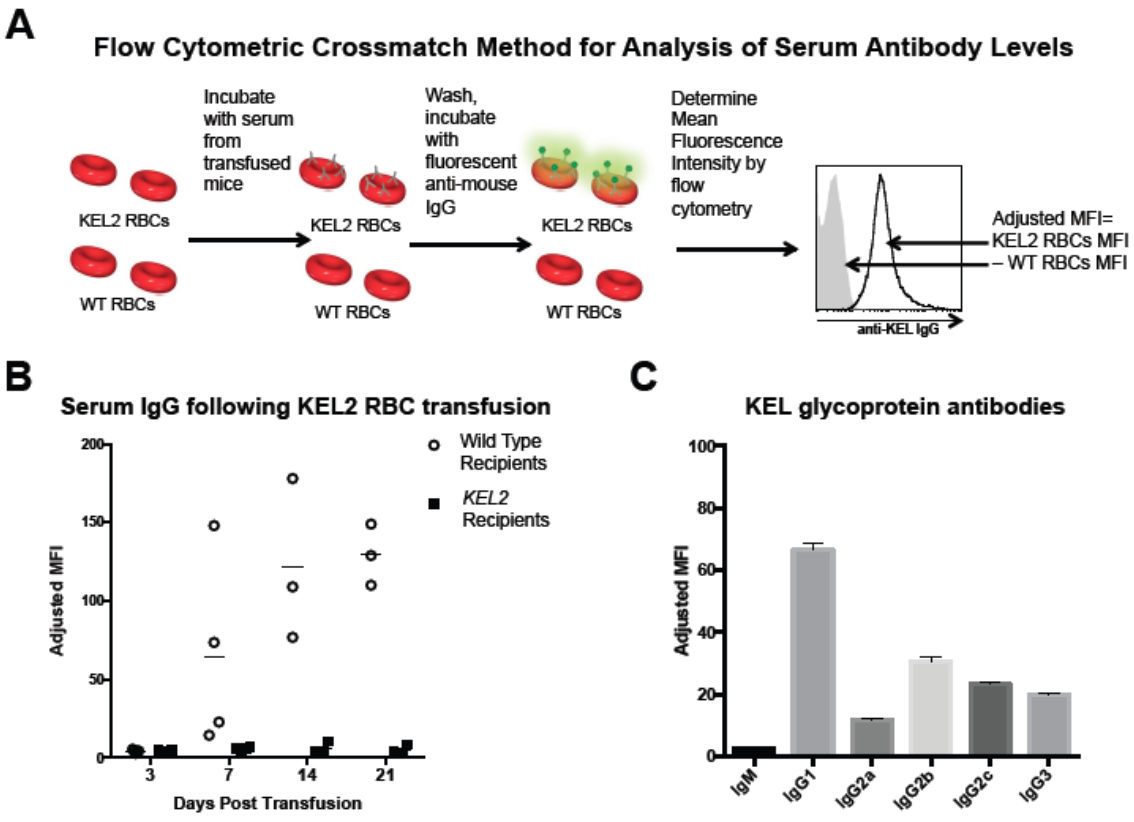
(solid line). These data are representative of 4 (A) or 5 (B) independent experiments, with 3-5 animals/group/experiment.; * $p<0.05$.

Figure 4.5: Levels of detectable anti-KEL IgG bound to transfused KEL2 RBCs decreases in immunized wild type and FcγR KO animals to 24 hours post-transfusion. A) Detectable IgG bound to transfused KEL2 RBCs in wild type, FcγR KO, or C3 KO animals passively transferred with polyclonal anti-KEL (solid line) or saline (shaded histogram); representative plots are shown. **B)** Compilation graphs of adjusted MFI of detectable IgG bound to KEL2 RBCs in wild type, FcγR KO, or C3KO animals transferred with polyclonal anti-KEL, with background signal from animals transferred with saline subtracted out. These data are representative of 5 independent experiments, with 3-5 animals/group/experiment.

Figure 4.6: Amount of detectable KEL glycoprotein on RBCs transfused into immunized wild type and FcγR KO animals decreases to 24 hours post-transfusion. A) Detectable KEL glycoprotein on KEL2 RBCs transfused into in wild type, FcγR KO, or C3 KO animals passively transferred with polyclonal anti-KEL (solid line) or saline (shaded histogram); representative plots are shown. **B)** Compilation graphs of the MFI of detectable KEL glycoprotein on KEL2 RBCs transfused into wild type, FcγR KO, or C3 KO animals, passively immunized with saline or polyclonal anti-KEL. These data are representative of 5 independent experiments, with 3-5 animals/group.

Figure 4.7: Decrease in detectable KEL glycoprotein beyond 10 minutes cannot solely be attributed to clearance of KEL2 RBCs with the highest antigen level. A) Following passive immunization and transfusion as previously described, clearance rates of DiI labeled KEL2 RBCs were calculated between time points. **B)** Changes in detectable KEL glycoprotein levels between time points was calculated by creating a gate which includes the cells from a particular time point and overlaying that gate onto the antigen level of the previous time point to determine the percent change**. C)** Representative bar graphs comparing the data from **A** and **B**. These data are representative of 5 independent experiments, with 3-5 animals/group/experiment. Error bars represent standard deviation and * represents statistical significance $p<0.01$.

Figure 4.1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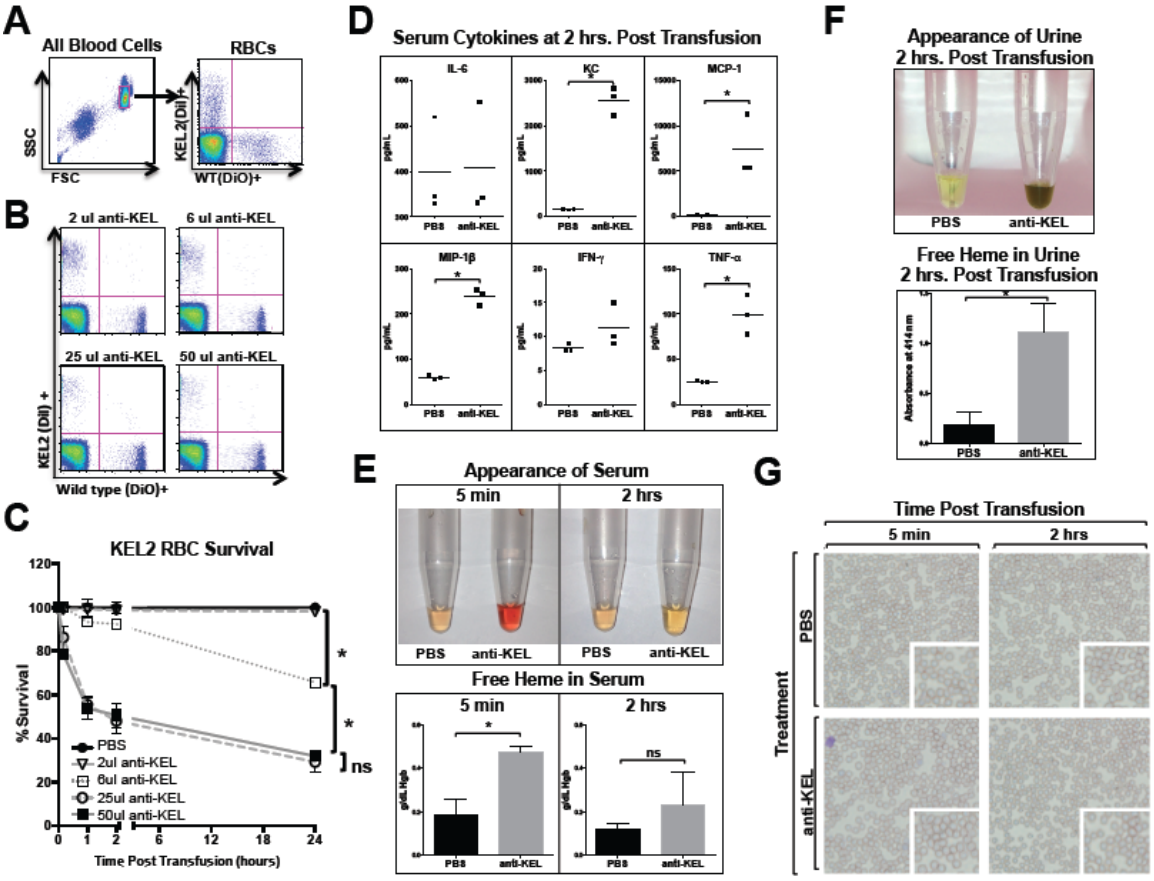
WT RBCs
Incubate with serum from transfused mice
KEL2 RBCs
WT RBCs
Wash, incubate with fluorescent anti-mouse IgG
KEL2 RBCs
WT RBCs
Determine Mean Fluorescence Intensity by flow cytometry
anti-KEL IgG
Adjusted MFI= KEL2 RBCs MFI – WT RBCs MFI
B
Serum IgG following KEL2 RBC transfusion
Adjusted MFI
Wild Type Recipients
KEL2 Recipients
Days Post Transfusion
C
KEL glycoprotein antibodies
Adjusted MFI
IgM
IgG1
IgG2a
IgG2b
IgG2c
IgG3

Figure 4.2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All Blood Cells
RBCs
SSC
FSC
KEL2(DiI)+
WT(DiO)+
B
2 ul anti-KEL
6 ul anti-KEL
25 ul anti-KEL
50 ul anti-KEL
KEL2 (DiI) +
Wild type (DiO)+
C
KEL2 RBC Survival
% Survival
PBS
2ul anti-KEL
6ul anti-KEL
25ul anti-KEL
50ul anti-KEL
Time Post Transfusion (hours)
ns
D
Serum Cytokines at 2 hrs. Post Transfusion
IL-6
KC
MCP-1
MIP-1β
IFN-γ
TNF-α
PBS
anti-KEL
E
Appearance of Serum
5 min
2 hrs
Free Heme in Serum
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Appearance of Urine
2 hrs. Post Transfusion
Free Heme in Urine
2 hrs. Post Transfusion
G
Time Post Transfusion
Treatment

Figure 4.3

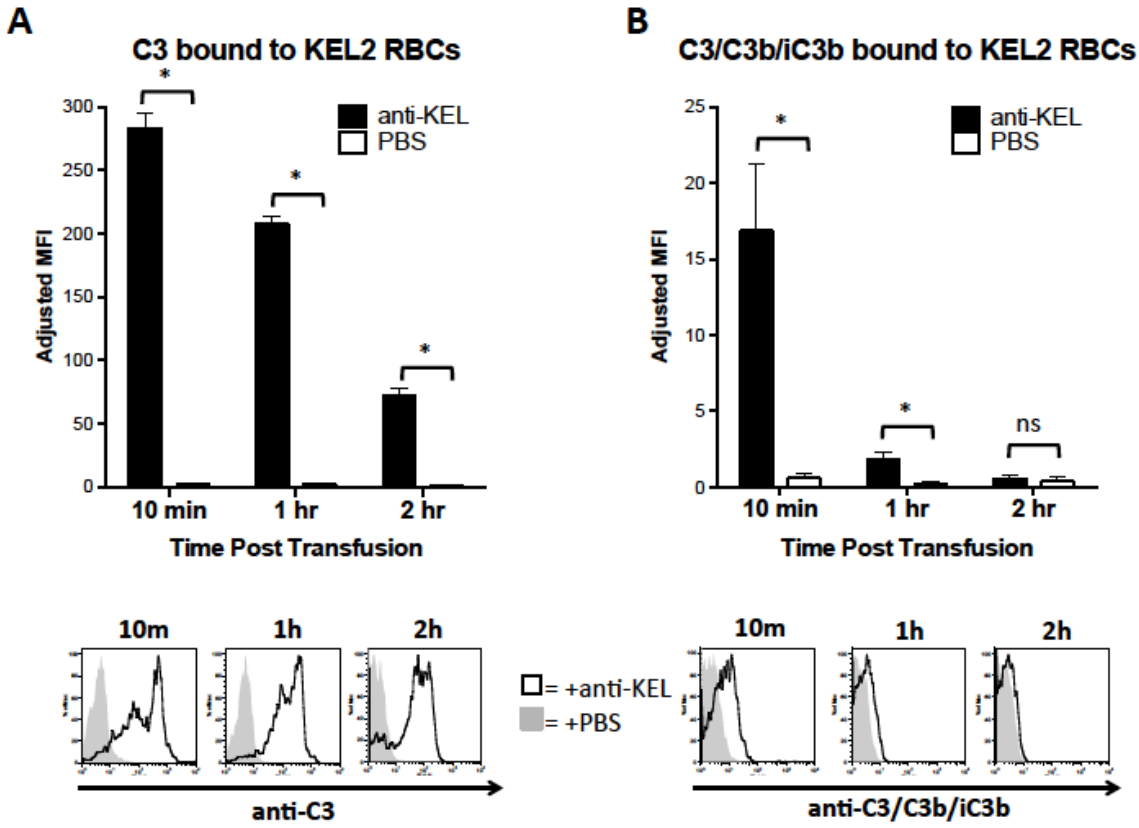

A
C3 bound to KEL2 RBCs
anti-KEL
PBS
Adjusted MFI
300
250
200
150
100
50
0
*
*
*
10 min
1 hr
2 hr
Time Post Transfusion
B
C3/C3b/iC3b bound to KEL2 RBCs
anti-KEL
PBS
Adjusted MFI
25
20
15
10
5
0
*
*
ns
10 min
1 hr
2 hr
Time Post Transfusion
10m
1h
2h
= +anti-KEL
= +PBS
anti-C3
10m
1h
2h
anti-C3/C3b/iC3b

Figure 4.4

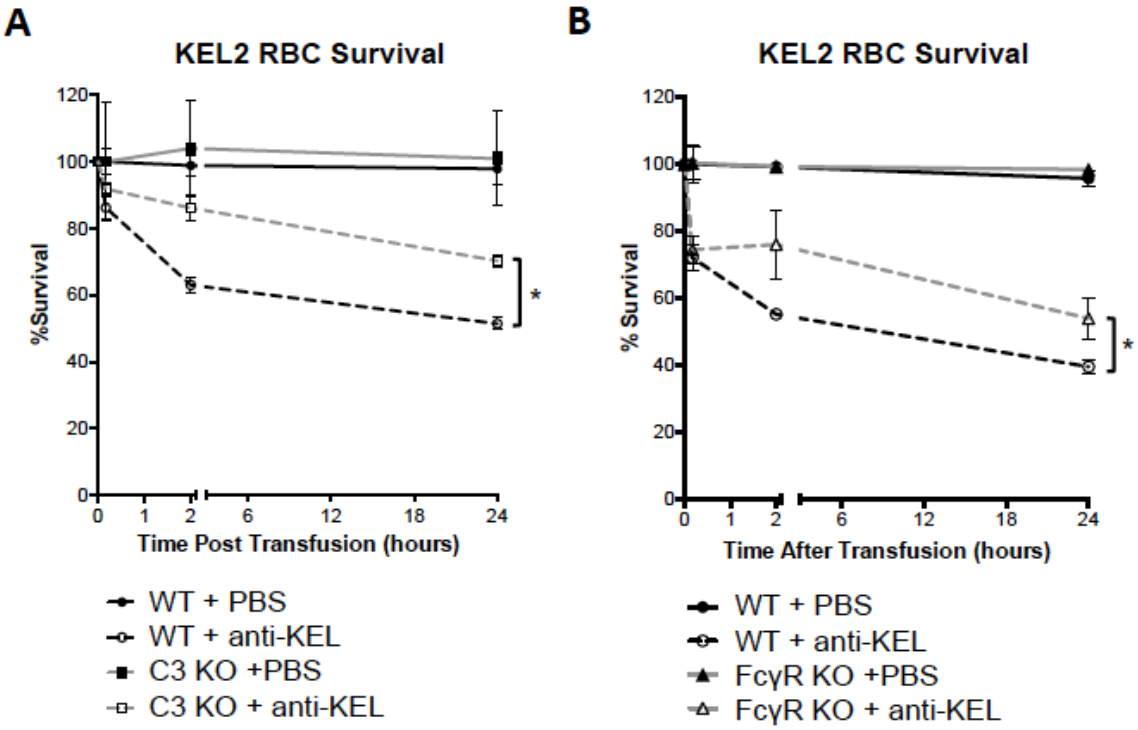

A
KEL2 RBC Survival
%Survival
Time Post Transfusion (hours)
WT + PBS
WT + anti-KEL
C3 KO +PBS
C3 KO + anti-KEL
*
B
KEL2 RBC Survival
% Survival
Time After Transfusion (hours)
WT + PBS
WT + anti-KEL
FcγR KO +PBS
FcγR KO + anti-KEL
*

Figure 4.5

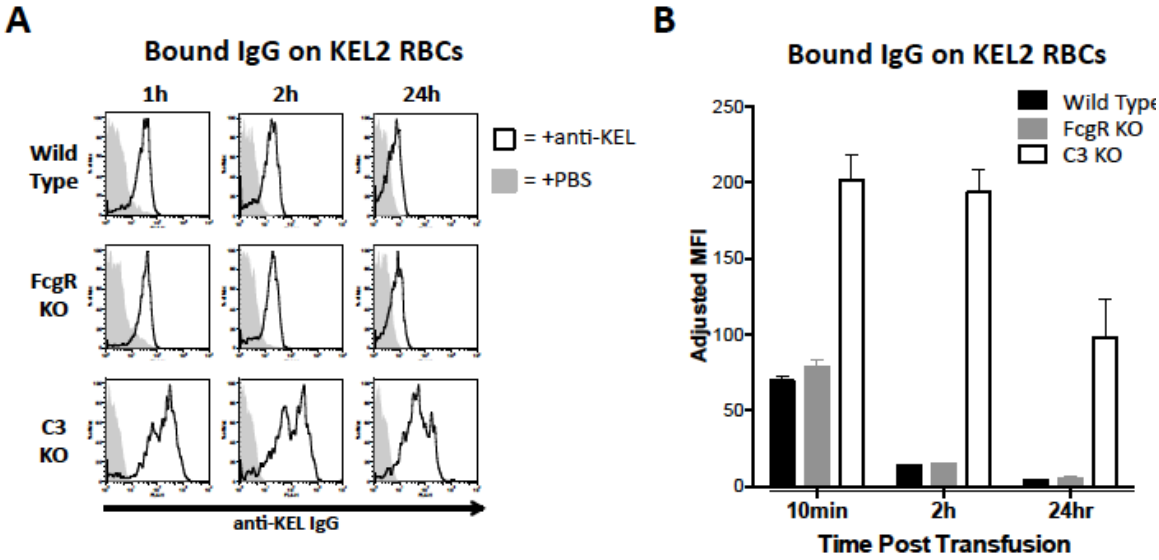

A
Bound IgG on KEL2 RBCs
1h
2h
24h
Wild Type
FcgR KO
C3 KO
= +anti-KEL
= +PBS
anti-KEL IgG
B
Bound IgG on KEL2 RBCs
Wild Type
FcgR KO
C3 KO
Adjusted MFI
250
200
150
100
50
0
10min
2h
24hr
Time Post Transfusion

Figure 4.6

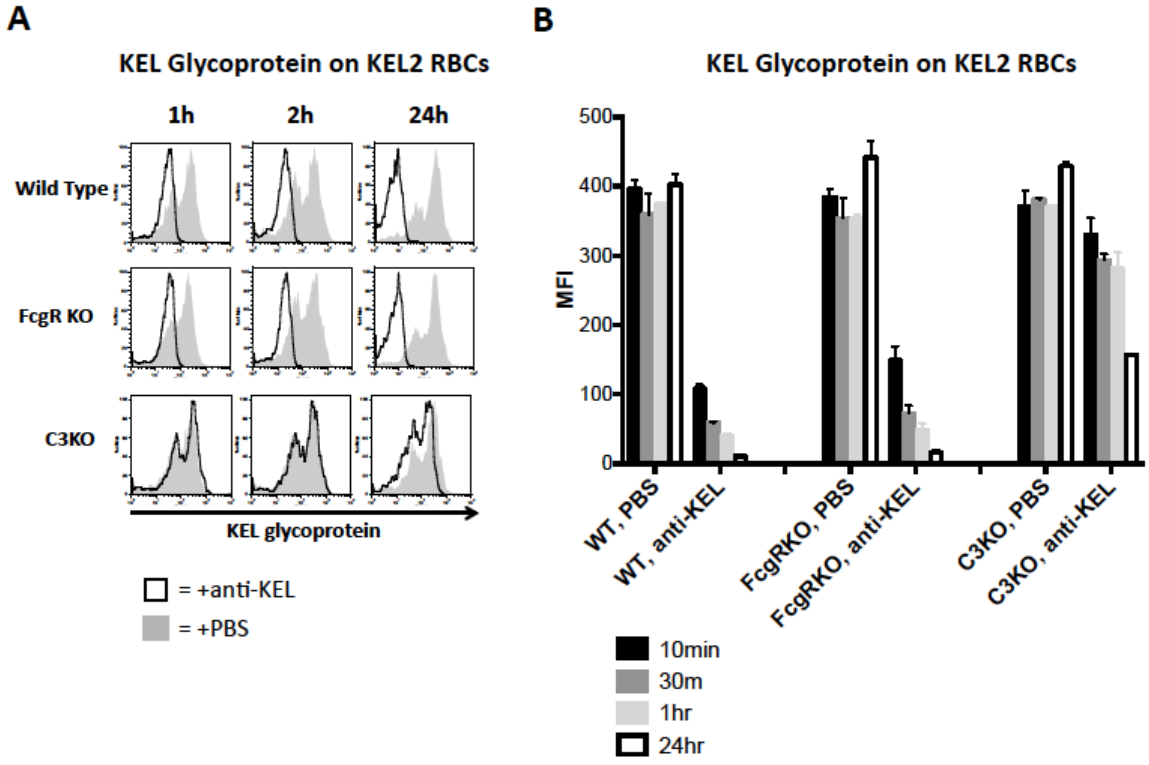
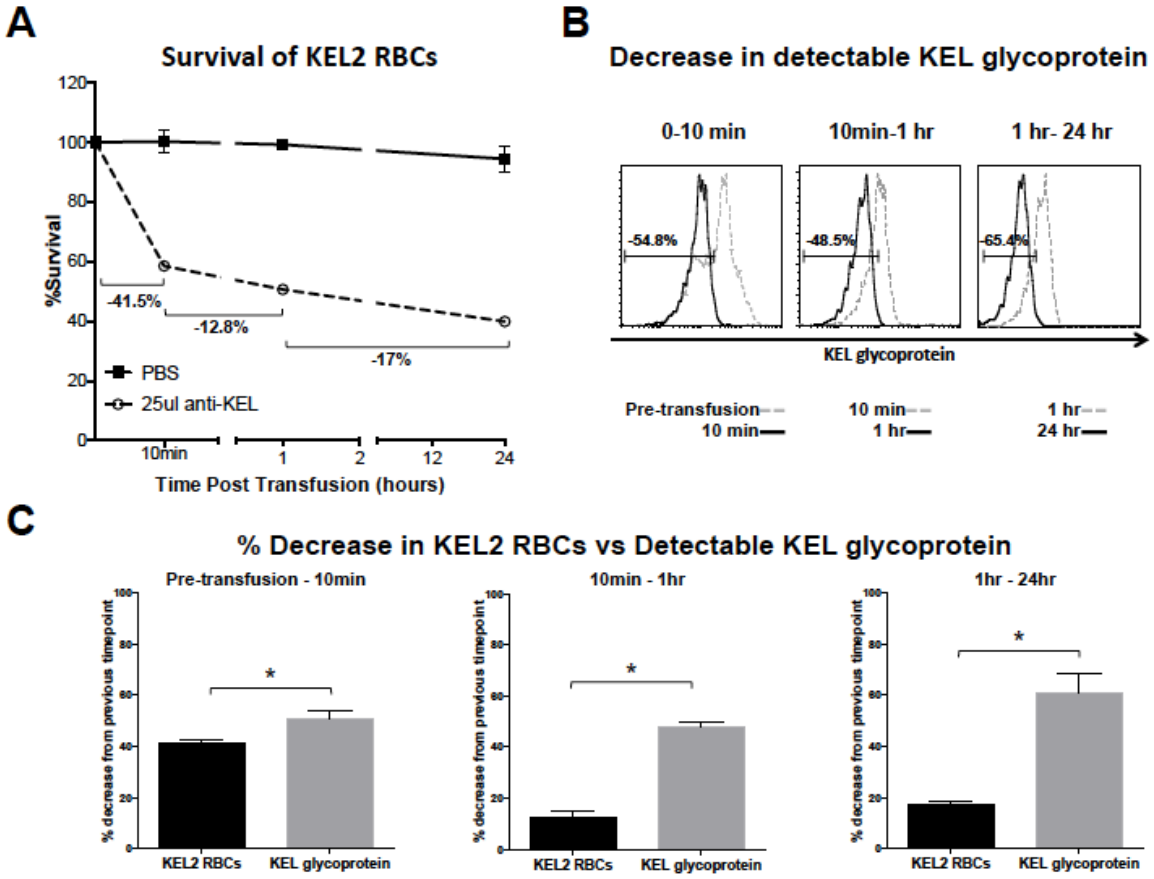

A
KEL Glycoprotein on KEL2 RBCs
1h
2h
24h
Wild Type
FcgR KO
C3KO
KEL glycoprotein
= +anti-KEL
= +PBS
B
KEL Glycoprotein on KEL2 RBCs
MFI
500
400
300
200
100
0
WT, PBS
WT, anti-KEL
FcgRKO, PBS
FcgRKO, anti-KEL
C3KO, PBS
C3KO, anti-KEL
10min
30m
1hr
24hr

Figure 4.7


A
Survival of KEL2 RBCs
%Survival
120
100
80
60
40
20
0
-41.5%
-12.8%
-17%
PBS
25ul anti-KEL
10min
1
2
12
24
Time Post Transfusion (hours)
B
Decrease in detectable KEL glycoprotein
0-10 min
10min-1 hr
1 hr- 24 hr
-54.8%
-48.5%
-65.4%
KEL glycoprotein
Pre-transfusion
10 min
10 min
1 hr
1 hr
24 hr
C
% Decrease in KEL2 RBCs vs Detectable KEL glycoprotein
Pre-transfusion - 10min
10min - 1hr
1hr - 24hr
% decrease from previous timepoint
*
KEL2 RBCs
KEL glycoprotein

Chapter 5

Alloantibodies to a paternally derived RBC Kell antigen lead to hemolytic disease of the fetus/newborn in a murine model

ABSTRACT

Exposure to non-self RBC antigens, either from transfusion or pregnancy, may result in alloimmunization and incompatible RBC clearance. First described as a pregnancy complication 80 years ago, hemolytic disease of the fetus and newborn (HDFN) is due to alloimmunization to paternally derived RBC antigens. Despite the morbidity/mortality of HDFN, women with or at risk for RBC alloimmunization have few therapeutic options. Given that alloantibodies to antigens in the KEL family are amongst the most clinically significant, we developed a murine model with RBC specific expression of the human KEL antigen to evaluate the impact of maternal/fetal KEL incompatibility. Following exposure to fetal KEL RBCs during successive pregnancies with KEL positive males, 21/21 wild type female mice developed anti-KEL alloantibodies; intrauterine fetal anemia and/or demise occurred in a subset of KEL positive pups born to wild type but not agammaglobulinemic mothers. Similar to what has been observed in humans, pregnancy associated alloantibodies were detrimental in a transfusion setting, and transfusion associated alloantibodies were detrimental in a pregnancy setting. This model, which is the first pregnancy associated HDFN model described to date, will serve as a platform to develop targeted therapies to prevent/mitigate the dangers of RBC alloantibodies to fetuses and newborns.

INTRODUCTION

Blood groups A,B, and O are well known to clinicians across all specialties. However, there are hundreds of other less well known blood group antigens on RBCs and other hematopoietic cells[1,2]. These antigens are capable of stimulating alloantibody formation in individuals whose immune system recognizes them as foreign, with subsequent antigen/antibody interactions potentially causing harm in settings including transfusion, pregnancy, and transplantation. Alloantibodies to such antigens on RBCs, including those in the Rh, KEL, Kidd, and Fy families, may lead to hemolytic transfusion reactions or hemolytic disease of the newborn (HDFN).

HDFN was first described in the 1930s, and realized to be an antibody mediated process a decade later[3]. Since that time, more than 50 antigens have been associated with HDFN, which affects more than 6/1000 live births[4,5]. In HDFN, IgG alloantibodies against blood group antigens cross the placenta and bind to RBCs in the fetal circulation, potentially resulting in hemolysis, reticulocytopenia, and fetal death in severe cases. Some women enter pregnancy with pre-existing RBC alloantibodies from transfusion, whereas other women becoming alloimmunized to foreign paternally derived RBC antigens present on the RBCs of the fetus during gestation/delivery. RBC phenotypic differences between partners are not routinely taken into consideration prior to conception and thus RBC alloimmunization and HDFN are potential risks in nearly all pregnancies. Surviving children may require simple or exchange RBC transfusion, IVIG, and/or

phototherapy; severely affected children may even be affected by developmental delay and cerebral palsy[6,7].

With the exception of polyclonal anti-D (RhoGam), there are no known therapies to prevent RBC alloimmunization or to mitigate the dangers of existing RBC alloantibodies. Since the introduction of anti-D, Rh(D) pregnancy associated alloimmunization has decreased by 95%[8]. In fact, anti-D is one of the most successful immunotherapies in use today. However, its mechanism of action remains ill defined. Furthermore, no monoclonal anti-D preparation has been deemed safe and effective enough to be FDA licensed for use in a pregnancy setting[9,10]. Limitations in the understanding of the mechanism of action of anti-D, as well as in the development of therapeutic options to prevent pregnancy associated RBC alloimmunization, is due largely to a lack of in vivo experimental models. The generation of transgenic animals with RBC specific expression of the human Rh(D) antigen has remained elusive, due in part to the genetic complexities of Rh(D).

The “Kell factor” was initially described half a century ago, following hydropic fetal complications[11] and fatal transfusion reactions[12]. It is now appreciated that the Kell factor is actually a family of antigens, with Kell being a glycoprotein with endopeptidase activity[2]. Multiple epitopes on the Kell protein have been defined as clinically significant antigens, including KEL1/KEL2, $Js^{a/b}$, and $Kp^{a/b}$[13]. Approximately 91% of Caucasians and 98% of African Americans lack the KEL1

antigen on their RBCs, thus putting them at risk of alloimmunization upon exposure to antigen positive RBCs, be it through transfusion or pregnancy. Today, KEL alloantibodies are a leading cause of antibody mediated transfusion and pregnancy associated morbidity/mortality[5,14-18].

To our knowledge, no animal model to date has been generated in which pregnancy associated RBC alloantibodies lead to adverse fetal outcomes. Limited knowledge about the RBC antigen systems of animals, in combination with lack of clinical significance of alloantibodies against these antigens, has contributed to the lack of model development. We have recently described a mouse model in which transgenic animals have RBC specific expression of the clinically significant human KEL glycoprotein antigen[19]. Herein, we demonstrate that paternally derived KEL antigens on fetal RBCs lead to maternal anti-KEL RBC alloimmunization through pregnancy/delivery, with subsequent pregnancies and transfusions being adversely affected by these boostable anti-KEL glycoprotein alloantibodies. This model thus lays the groundwork to investigate pregnancy associated alloantibody formation and subsequent RBC clearance mechanisms, as well as targeted therapeutic strategies to prevent or mitigate the dangers of pregnancy associated alloantibodies.

MATERIALS AND METHODS

Mice

Mice transgenic for the KEL human RBC antigen (specifically the KEL2 or Cellano antigen with mid levels of expression near 1300 copies/RBC, previously described as "KEL2B") were generated as previously described [19], utilizing a B-globin promoter. C57BL/6 mice were purchased from the National Cancer Institute (Fredricksburg, ML); MuMT, uGFP, and IgHa (IgHa x Thy1.1 x GPI) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). For breeding experiments, C57BL/6 or MuMT females at 2-3 months of age were mated with heterozygous KEL or homozygous uGFP males; all protocols were approved by the Emory University Institutional Animal Care and Use Committee.

Blood collection, labeling, and transfusion

Donor blood was collected in the anticoagulant preservative solution ACD (BD, Fisher Scientific). After being washed twice with phosphate buffered saline, KEL RBCs were labeled with the lipophilic dye chloromethylbenzamido 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI) control C57BL/6 RBCs were labeled with 3,3'-dihexadecyloxacarbocyanine perchlorate (DiO) as previously described[20-23] (Molecular Probes, Eugene, OR). C57BL/6 recipients were transfused via lateral tail vein with a mixture of 50-75 µL of KEL and C57BL/6 RBCs, diluted with saline to a total volume of 300 µL. Post-transfusion RBC recovery was determined by flow cytometry at multiple time

points post-transfusion. A subset of recipients were pretreated with 100 μg of poly (I:C) (Amersham, Piscataway, NY) 4 hours prior to transfusion.

Hematologic evaluation, flow cytometry and antibody generation/detection

Wright Giemsa stained smears were evaluated using an Olympus BX41 microscope; cells were quantitated using APC Calibrite beads (BD Biosciences) and stained with thiazol orange. Serum bilirubin was evaluated by QuantiChrom Assay (BioAssay Systems, Hayward, CA). RBC bound anti-KEL was detected on transfused DiI labeled RBCs using an IgG secondary reagent conjugated to allophycocyanin (APC). To detect serum anti-KEL, serum was titrated and crossmatched with KEL or control C57BL/6 RBCs, and APC-conjugated goat anti mouse IgG (BD Biosciences) or HRP conjugated goat anti-mouse IgG subtypes (Bethyl Laboratories, Montgomery, TX) plus anti-HRP Cy5.5 (Jackson Immunoresearch Laboratories, West Grove, PA) were utilized. An adjusted mean fluorescence intensity (MFI) was determined by subtracting background binding signal with control RBCs from that of target RBCs.

For experiments involving fetal livers, APC-conjugated CD4, CD3, B220, CD11b, CD11c, and CD41 were used to exclude non RBC precursors; FITC-conjugated TER119 and PE-conjugated CD71 were used to identify fetal liver erythroid precursor populations[24,25]; polyclonal anti-KEL generated in IgHa recipients, biotin-conjugated mouse anti-mouse IgG1a (BD Biosciences), PerCP-Cy5.5-conjugated Strepavidin (Ebioscience, San Diego, CA), and anti-mouse Igs (BD

Biosciences) were also used. FITC-conjugated TER119 and APC-conjugated CD44 (BD Biosciences) were used to stain bone marrow, with CD45 and CD11 exclusion gates. All results were acquired on a BD FacsComp flow cytometer, and analyzed using FloJo Software (Treestar; Ashland, OR).

Tissue antigen detection by RT-PCR

KEL animals were fully myeloablated with split doses of total body irradiation totaling 900 cGY, and transplanted with 5 x 10^6 splenocytes from C57BL/6 animals. After ensuring full RBC engraftment, organs were harvested, placed into RNAlater Stabilization Reagent (Qiagen, Valencia, CA) and homogenized; RNA was isolated by phenol-chloroform extraction using TRIzol Reagent (Invitrogen, Carlsbad, CA). An RNeasy Mini Kit (Qiagen) was used to digest contaminating DNA and to isolate bone marrow RNA. For PCR, cDNA was synthesized using AccuScript Reverse Transcriptase (Stratagene, Santa Clara, CA) and amplified using PCR Master Mix (Promega, Madison, WI). Primers specific for Kell (5'–GGGGGATCCGCCACCATGGAAGGTGGGGACCAAAGTG and 3'–TTGGAACAGAAGCAGAAAGGAA) and control primers specific for mouse G3PDH (5'–ACCACAGTCCATGCCATCAC and 3'–TCCACCACCCTGTTGCTGTA) were used for amplification.

MRI imaging

All MRI image data were acquired at 9.4 T on a BioSpec 94/20 spectrometer (Bruker; Billerica, MA), using a radio frequency (r.f.) volume coil with 72 mm inner

diameter. After isofluorane anesthesia, T2 weighted (T2W) images were obtained using a fast spin-echo sequence with the following parameters: TR/TE = 3800/84 ms, FOV = 51.2 × 36 mm^2, matrix = 256 x 180, resolution = 200 × 200 μm^2 in plane, slice thickness = 0.8 mm with 0.2 mm inter slice gap, average = 12, rapid acquisition with relaxation enhancement (RARE) factor = 8. MR images were generated using the Paravison 5.1 software package (Bruker BioSpec, Billerica, MA).

Statistical analysis

Statistical analysis and graphing were performed using Graph Pad Prism software (San Diego, CA). Statistically significant differences between 2 groups were compared using an unpaired t test, and differences between 3 or more groups were calculated by a one way analysis of variance with a Tukey post test. A Fisher's exact test used to evaluate differences between proportions. In all instances, statistical significance was defined as $p<0.05$.

RESULTS

Smaller successive litters with fewer KEL positive pups are born to wild type females mated with KEL males

Wild type C57BL/6 females were bred with KEL heterozygous males, and pregnancy outcomes documented. In a total of 59 pregnancies, the mean number of live births was 6.3 (1st litters), 5.3 (2nd litters), and 4.2 (3rd litters) (**Figure 5.1A**). The number of pups alive at weaning also declined with successive pregnancies (**Figure 5.1B**), as did the percentage of KEL positive pups alive at weaning (**Figure 5.1C**, mean 53.1% in 1st litters, compared to 19.3% in 3rd litters). The observed decrease in litter size wasn't an artifact of advanced maternal age, as 16 control pregnancies between KEL heterozygous females and wild type C57BL/6 males showed no statistically significant declines in litter sizes at birth (**Figure 5.1D**) or weaning (**Figure 5.1E**) over time. Furthermore, there were no statistically significant differences in percentages of KEL positive pups with successive pregnancies in these control animals (**Figure 5.1F**).

All pale/hydropic pups are KEL positive by PCR

Approximately 10% of pups born in 3rd and beyond litters to wild type females bred with KEL males were stillborn, pale, and/or hydropic appearing (**Figure 5.2A** depicts littermates born in a 3rd litter). All stillborn pups were screened for RBC expression of the human KEL antigen by PCR (**Figure 5.2B**). Documentation of the degree of anemia in stillborn pups or those who died within

hours of birth was inherently difficult; however, **Figure 5.2C** documents anemia in the pale pup shown using trucount beads (small boxes) and counting TER 119 positive events (large boxes). This pale pup also had a subtle, non-statistically significant increase in thiazol orange reticulocyte staining at birth (**Figure 5.2E,F**), with an increase in thiazol orange staining noted in this and other pale KEL positive pups compared to KEL negative pups within the first week of life and returning to baseline by weaning. There were no statistically significant differences in serum bilirubin between KEL positive and KEL negative pups (mean 1.5 vs 1.3 mg/dL), though one severely anemic pup did have a level above the reference range. Blood smears of KEL positive pups born to multiparous C57BL/6 females (**Figure 5.2G**) show Howell-Jolly bodies, nuclear extrusion, polychromasia, and basophilic stippling.

Although 100% (10 of 10) of swollen/hydropic appearing pups were KEL positive by PCR, not all stillborn pups or those who died between birth and weaning were KEL positive. Cesarean sections of two mothers at 18-20 days of gestation revealed ongoing reabsorption of deceased fetuses, with surrounding areas of fibrosis involving fetuses in close uterine proximity (data not shown), suggesting a “bystander”effect. To allow for longitudinal evaluation of pups during pregnancy, MRI was completed on pregnant mothers using a 9.4T imaging machine. Although classic signs of “hydrops” could not be observed in the pups of the mothers imaged likely due in part to resolution considerations, fewer pups were observed in 3rd compared to 1st litters (**Figure 5.2H).**

KEL expression is RBC specific and present on early RBC precursors

The KEL transgenic animals were made utilizing a B-globin specific promoter[19], and thus expression of the KEL antigen would be predicted to be RBC specific. This B-globin promoter has been shown to be expressed around day 14[26]; thus, KEL expression was investigated on RBC precursors from post-conception (p.c.) day 13 through delivery. Fetal livers were stained with TER119 ,CD71, and anti-KEL, with exclusion gates. As early as p.c. day 13, KEL expression was detected on all stages of RBC precursors (**Figure 5.3A, B, C**). To further characterize KEL expression, bone marrow was stained with TER119, CD44, and anti-KEL, with exclusion gates[27,28]. All evaluated RBC precursors in the bone marrow also had detectable KEL expression (**Figure 5.3D**), though this KEL expression was not uniform throughout RBC precursor maturation. In addition to fetal liver and BM RBC precursors, all circulating TER119 positive but not TER119 negative cells had detectable KEL expression (**Figure 5.3E**).

To evaluate whether KEL was expressed in non-hematopoietic tissues[29], organs were harvested and evaluated for KEL by RT-PCR. As predicted, KEL expression was observed in the bone marrow but not in the spleen, kidney, liver, heart, and lung (**Figure 5.3F**). Studies were also undertaken in KEL transgenic animals that been transplanted with C57BL/6 marrow under fully myeloablative conditions, to eliminate the possibility of KEL reticulocyte contamination. Following engraftment and after confirming a lack of circulating KEL RBCs, organ

RT-PCR was completed. As expected, the KEL transgene, which is driven by the human B-globin promoter cassette[19,26], was RBC specific with a lack of KEL expression on the organs or in the bone marrow of transplanted animals.

Anti-KEL is generated by wild type females mated with KEL males

To test the hypothesis that the immune response hypothesized to occur in response to fetal/maternal RBC transfer is antibody mediated, serum from wild type C57BL/6 females mated with KEL males was evaluated for anti-KEL glycoprotein antibodies prior to and at weekly intervals during pregnancy and after delivery. Consistent with the finding that no pregnant female had detectable fetal RBCs in circulation until after delivery (**Figure 5.4A-C**), <5% (2/42) developed anti-KEL glycoprotein antibodies during their first pregnancies; this antibody was low titer and litter sizes were normal. However, essentially all mice had detectable anti-KEL glycoprotein antibodies after their 3rd pregnancies (**Figure 5.4D-G;** mean adjusted MFI 1.3 in nulliparous versus 124.7 in multiparous females). An increase in maternal antibody anti-KEL titer was observed with sequential pregnancies/deliveries (**Figure 5.4H)**; no female with an anti-KEL IgG titer below 1:32 had any affected anemic pups. Taken together, these data suggest fetal/maternal RBC transfer of RBCs expressing a paternally derived antigen (KEL) foreign to the mother leads to maternal alloimmunization, with the largest detectable RBC transfer occurring around the time of delivery.

Anti-KEL glycoprotein IgG crosses the placenta and binds to the RBCs of KEL positive pups

To determine whether anti-KEL crossed the placenta and bound to KEL RBCs in KEL positive pups, peripheral blood samples were taken at weaning from pups born to alloimmunized wild type females, and stained with anti-mouse IgG in a direct antiglobulin test or DAT (**Figure 5.5A**). The binding was KEL specific, as the antibody did not bind to antigen negative RBCs above levels of secondary antibody alone, and KEL negative pups had largely negative DATs. No anti-KEL was detected in the circulation via indirect antiglobulin test (IAT) in KEL positive pups (**Figure 5.5B**) presumably due to RBC saturation; consistent with this, KEL negative pups had positive IATs. Additional studies were completed using flow based crossmatch to evaluate the ability of maternal anti-KEL IgG subtypes to cross the placenta. Anti-KEL IgG1, IgG2a, IgG2b, and IgG2c were detected in alloimmunized multiparous mothers, with all IgG subtypes also being detected in the neonatal circulation at the time of birth (**Figure 5.5C**).

Decreased numbers of newborn liver RBC precursors are detected in KEL positive pups born to alloimmunized mothers

To further investigate the in utero effect of maternal anti-KEL IgG, RBC precursors from newborn livers were evaluated on surviving newborn pups using trucount beads. In 3 of 4 pregnancies, KEL positive pups born to alloimmunized mothers had significantly decreased numbers of all stages of liver RBC precursors, compared to their KEL negative littermates (**Figure 5.5D**, left

column). Furthermore, these liver RBC precursors were reactive by direct antiglobulin test (**Figure 5.5D**, right column). Although the exact mechanism of fetal anemia in this model remains under investigation, suppression of erythropoiesis during in utero development likely plays a contributory role.

MuMT females bred multiple times with KEL males have healthy pups

To further evaluate the role of maternally derived anti-KEL alloantibody in the observed fetal demise, MuMT females that lack mature B cells were bred with KEL males three times and litter statistics were evaluated. Consistent with their phenotype, no anti-KEL could be detected in the serum of these MuMT females after any pregnancy. Furthermore, the MuMT females had a similar number of pups in 1st (mean 8) compared to 3rd litters (mean 8.5) with approximately 50% KEL positive pups (**Figure 5.5E**), in contrast to the decrease in litter size and percent KEL positivity observed over time in wild type females bred with KEL males (**Figure 5.1A**). In combination, these data lend further support that the anti-KEL alloantibody generated by wild type females bred with KEL males likely plays a central role in the observed fetal/neonatal demise.

Anti-KEL generated through pregnancy leads to clearance of transfused KEL RBCs and a pro-inflammatory serum cytokine storm

To investigate whether anti-KEL generated in pregnancy was capable of leading to clearance of KEL RBCs, a transfusion based approach was taken. For these

experiments, KEL RBCs were labeled with the lipophilic dye Di, and control wild type C57BL/6 RBCs were labeled with the lipophilic dye DiO. A mixture of KEL and control RBCs was transfused into C57BL/6 females who had previously been pregnant 3 or more times with KEL positive pups and who had significant levels of anti-KEL glycoprotein IgG but minimal levels of anti-KEL glycoprotein IgM; control recipients were nulliparous females. Clearance of the KEL positive cells was determined by comparing the percentage of circulating KEL DiI labeled RBCs to C57BL/6 DiO labeled RBCs over time (**Figure 5.6A**). In 3 of 3 experiments (n=21 animals total), rapid initial clearance of "incompatible" KEL RBCs was observed, followed by persistent circulation of a subset of KEL RBCs (**Figure 5.6B,** solid circles). In contrast, control nulliparous animals did not demonstrate preferential clearance of transfused DiI labeled KEL RBCs compared to wild type RBCs (**Figure 5.6B,** open circles). The clearance observed in alloimmunized females was presumably antibody mediated, as transfused KEL RBCs had significant levels of anti-KEL IgG bound within 10 minutes post-transfusion (**Figure 5.6C**).

Within 2 hours of transfusion of "incompatible" KEL RBC transfusions, alloimmunized animals became transiently hunched and ill appearing. In a compilation of 3 experiments (n=9 alloimmunized compared to 9 controls), elevations of serum cytokines were observed with respect to serum levels of IL-6 (**Figure 5.6D**), KC (**Figure 5.6E**), and MCP-1 (**Figure 5.6F**). In addition, elevations were observed in serum levels of MIP-1B (mean 150 vs 19.7 pg/mL,

p<0.05) and in TNF-α (mean 79.8 vs 53.5 pg/mL, p<0.05); no significant differences were observed in serum levels of IFN-γ. However, it cannot be inferred that the mechanisms leading to clearance of transfused incompatible KEL RBCs are necessarily the same as that observed during the development of KEL positive pups in the uterus of alloimmunized mothers.

Transfusion associated KEL alloimmunization is boostable, and leads to adverse pregnancy outcomes

Multiple factors in humans prevent a direct juxtaposition between the dangers to fetuses of RBC alloantibodies generated through pregnancy compared to those generated through prior transfusion. However, either method of exposure and antibody acquisition can be detrimental to the human fetus[5]. To investigate whether the method by which a female is exposed to an RBC antigen determines the clinical significance or subtype of the alloantibody in this reductionist murine system, wild type C57BL/6 animals were transfused every 2-3 weeks with KEL RBCs in the presence of the double stranded RNA poly (I:C); anti-KEL responses were evaluated by flow cytometric crossmatch 2 weeks after each transfusion. The distribution of anti-KEL IgG subtypes was similar but not identical between animals immunized through transfusion compared to pregnancy (**Figure 5.7A**); recent studies in our laboratory have more fully characterized the time course of IgG and IgM antibody responses post-transfusion[30].

In 3 of 3 experiments (n=30 recipients total), 100% of recipients made detectable anti-KEL glycoprotein Igs after an initial transfusion, which increased further in response to subsequent transfusions (**Figure 5.7B**). This boostable response was not simply due to a continued increase in response after the initial transfusion, as antibody responses peaked between 14-21 days after the initial transfusion; furthermore, this boostable response was not dependent on poly (I:C) pretreatment, as animals transfused in the absence of poly (I:C) also had a boostable anti-KEL response[30].

To determine whether transfusion induced anti-KEL alloantibodies were detrimental to KEL positive fetuses, wild type C57BL/6 females who had previously been transfused 3 times with KEL RBCs in the presence of poly (I:C) were bred with KEL positive males, and pregnancy outcomes were compared to pregnancy outcomes in control females never previously transfused or pregnant. Females immunized through transfusion had smaller litters compared to non-immunized females (**Figure 5.7C,** 5.5 vs 6.3 pups), fewer total pups alive at weaning per litter (**Figure 5.7D,** 2.6 vs 5.9 pups, $p<0.05$), and fewer KEL positive pups (**Figure 5.7E,** 22.6 vs 53.1%, $p<0.05$). Hydropic appearing pups, all documented to be KEL positive by PCR, were noted in a minority of deliveries. Thus, similar to what has been described in humans, transfusion induced anti-KEL alloimmunization is detrimental to KEL positive fetuses, as is pregnancy induced anti-KEL alloimmunization.

DISCUSSION

Herein we have described what, to our knowledge, is the first animal model of pregnancy induced RBC alloimmunization to a paternally derived, clinically significant RBC antigen (KEL). In this model, the human KEL antigen is present on transgenic murine RBCs, and fetal/maternal blood transfer during pregnancy and delivery leads to anti-KEL glycoprotein alloantibodies. These anti-KEL alloantibodies cross the placenta and bind to KEL expressing RBCs and RBC precursors in the fetal liver and the peripheral circulation of fetuses and neonates. The end result of anti-KEL RBC binding includes intrauterine fetal demise or hydropic stillborn pups; a subset of KEL positive pups born to mothers with low titers of anti-KEL survive to weaning. Control MuMT females, incapable of making anti-KEL glycoprotein antibodies, have unaffected pups when bred with KEL transgenic males, thus lending further support to the antibody mediated nature of the observed fetal demise.

KEL is known to be one of the most clinically significant RBC antigens in humans, both from a transfusion and HDFN perspective. In humans, anti-KEL1 antibodies lead to clearance of KEL positive RBCs and fatal hemolytic transfusion reactions[2]; in our murine model, anti-KEL antibodies also lead to clearance of transfused KEL positive RBCs, with a pro-inflammatory cytokine storm. In humans, KEL is expressed on early RBC precursors with anti-KEL1 antibodies leading to in utero suppression of erythroid progenitor cells and potentially severe HDFN[17,31]. In our transgenic murine model, human KEL is

expressed on murine RBC precursors as early as p.c. day 13, with anti-KEL antibodies also leading to in utero suppression of KEL erythroid progenitors. Of note, murine KEL is also expressed on RBCs during early during fetal development[27], though the antibodies generated in wild type C57BL/6 females after exposure to fetal murine RBCs expressing the human KEL antigen are reactive with the human but not the murine KEL antigen. Consistent with suppression of erythropoiesis as a contributing factor to fetal anemia and HDFN, KEL positive pups born to alloimmunized mothers lacked significant reticulocytosis or hyperbilirubinemia at the time of birth. However, these pups developed a compensatory reticulocytosis soon after birth, similar to that observed in human infants born to alloimmunized mothers.

Assay limitations prevented visualization of the fate of circulating KEL fetal cells in our model while the pups were in utero, though transfusion of “incompatible” KEL RBCs allowed for an alternate method of visualization. 50% of KEL RBCs transfused into females alloimmunized through pregnancy bound antibody, cleared rapidly, and resulted in a pro-inflammatory serum cytokine storm, with the other 50% remaining in circulation. This lack of complete KEL RBC clearance was not due to insufficient antibody, as similar observations were made in experiments in which large amounts of polyclonal anti-KEL glycoprotein Igs were passive transferred to naïve animals prior to KEL RBC transfusion (data not shown). Similar passive transfer experiments have revealed an antibody threshold below which no RBC clearance occurs, and likely explaining the

survival of a subset of KEL positive pups born to alloimmunized mothers. Determination of the mechanism of action of incompatible RBC clearance (and clearance resistance) in both transfusion and pregnancy settings is ongoing.

Although our described model is the first animal model of clinically significant RBC alloantibodies generated through pregnancy, rabbit models dating back to the 1950s have demonstrated the dangers of maternal RBC alloantibodies[3,32,33]. A model developed in the 1990s documented fetal hydrops, anemia, and reticulocytosis in response to maternal RBC antibody bound to fetal RBCs[34,35]. This rabbit model significantly increased knowledge about placental antibody transfer and effect on RBCs, though maternal alloantibody could be stimulated only through repeat immunizations with complete and incomplete freund's adjuvant mixed with the antigen. Therefore, the process of antibody formation through pregnancy to a clinically significant human RBC antigen has never been studied in an animal model.

Polyclonal anti-D is perhaps one of the most successful targeted immumodulatory therapies in existence[8,36], yet its mechanism of action remains elusive[8,37,38]. Many monoclonal anti-D antibodies have been generated and characterized in depth[10], though none to date have proven as efficacious as polyclonal anti-D at preventing Rh(D) alloimmunization. The lack of a mechanistic understanding of polyclonal anti-D is due, at least in part, to a lack of a reductionist model system in which to test hypotheses. In addition to the

polyclonal anti-KEL glycoprotein preparation that can be generated through transfusion or pregnancy, a number of monoclonal preparations targeting epitopes on the human KEL glycoprotein are already in existence. Thus, the newly described KEL system will offer the opportunity to test the abilities and mechanisms of such antibodies in preventing alloimmunization, with the realization that these findings may be applicable to some but not all human RBC antigens.

Limitations to the current studies must always be considered. This KEL model more closely resembles the human scenario of Rh(D) than the human KEL1/KEL2 scenario in the sense that the paternally derived foreign antigen (human KEL glycoprotein) is absent from the RBCs of the mother. However, the HDFN and antibody characteristics in this model are more similar to those described in the human KEL system. Other considerations include difficulties in fully visualizing and evaluating fetal KEL positive RBCs in the maternal circulation, as well as differences in murine and human fetal/maternal interfaces[39,40]. Although the fate of transfused RBCs into alloimmunized females was evaluated as a surrogate, differences in circulatory half life and clearance patterns between fetal and adult KEL RBCs cannot be ruled out. Furthermore, differences in antibody mediated clearance patterns based on RBC dose and KEL antigen density on RBCs must also be considered, though circulating RBCs in adult transgenic mice all appear to have similar levels of KEL antigen expression. Another consideration is that the males utilized in this study were

KEL heterozygous, allowing for comparison studies between KEL positive and negative littermates. It cannot be ruled out, however, that alloimmune responses may be different with KEL homozygous RBCs. Lastly, ongoing studies involving intrauterine fetal imaging and autopsy may shed further light on the timing of intrauterine fetal death and fetal pathology, including the possibility that fetal resorption, in addition to fetal/maternal hemorrhage, may be contributing to maternal alloimmunization.

In sum, we have described the first animal model of RBC alloantibodies generated through pregnancy, against the human KEL RBC antigen. The antibody response, which can be generated not only through pregnancy/delivery but also through transfusion, is boostable and clinically significant in both settings. This model thus provides a platform to study not only the induction, placental transfer, and consequences of RBC alloantibodies, but also potential therapeutics and their mechanism(s) of action. Long term translational goals of this work include decreasing the dangers of RBC alloantibodies to developing fetuses and neonates, through targeted maternal immunomodulatory therapies.

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FIGURE LEGENDS

Figure 5.1: Smaller successive litters with fewer KEL positive pups are born to wild type females mated with KEL males. (**A**) The number of pups alive at birth, (**B**) number of pups alive at weaning, and (**C**) percentage of KEL positive pups in 1st and 3rd litters of wild type females mated with KEL males. (**D**) The number of pups alive at birth, (**E**) number of pups alive at weaning, and (**F**) percentage of KEL positive pups in 1st and 3rd litters of control KEL females mated with wild type males. Data in A,B,C are a compilation of 40 total pregnancies; data in D,E,F are a compilation of 16 total pregnancies; *$p<0.05$.

Figure 5.2: A subset of pups born to multiparous wild type females mated with KEL males are stillborn or pale. (**A**) Photograph of a representative pink KEL negative and pale KEL positive pup, hours after birth to a multiparous (3rd litter) wild type female mated with a KEL male. (**B**) Representative KEL specific PCR. (**C**) Flow cytometric analysis of the blood of the pups shown, with 1000 true count beads and RBCs gated; (**D**) TER119 positivity of gated RBCs. (**E**) Thiazole orange reticulocyte staining and (**F**) TER119 staining of the blood of the same pups. (**G**) Blood smear of representative KEL negative and KEL positive pups. (**H**) MRI of 1st (left) or 3rd (right) pregnancies of representative wild type females bred with KEL males.

Figure 5.3: KEL is expressed on all RBC precursors in the fetal liver and bone marrow of transgenic animals, in an RBC specific fashion. (**A-C**) Fetal liver cells from KEL transgenic fetuses, with anti-KEL staining (solid) or secondary only staining (shaded) of RBC precursor populations (I=primitive erythroid progenitor cells, II=proerythroblasts and early basophilic erythroblasts, III=later basophilic erythroblasts, IV=chromatophilic and orthochromatophilic erythroblasts, and V=late orthrochromatophlic erythroblasts and reticulocytes) (**D**) Bone marrow from KEL transgenic animals, with anti-KEL staining (solid) or secondary only staining (shaded) of RBC populations (I=proerythroblasts, II=basophilic erythroblasts, III=polychromatic erythroblasts, IV=orthochromatic erythroblasts, V=reticulocytes, VI=mature RBCs). (**E**) Anti- KEL staining of peripheral blood cells. (**F**) Bone marrow and organs of a KEL positive animal were evaluated for KEL expression by PCR. Results are from 2 independent experiments, with 1-2 mice/group.

Figure 5.4: Fetal RBCs can be detected in the maternal circulation after delivery, with anti-KEL RBC antibodies detectable in the serum of wild type females after multiple pregnancies with KEL positive males. (A) Unstained RBCs from a nulliparous wild type female. (**B**) Unstained RBCs from a homozygous uGFP male. (**C**) Unstained RBCs from a representative wild type female bred with a homozygous uGFP male, p.c. day 7, p.c. day 14, and hours after delivery. (**D,E)** Serum from nulliparous or multiparous animals was crossmatched with TER119 positive RBCs. (**F**) Representative flow crossmatch

against wild type (shaded) or KEL (solid) RBCs, from a nulliparous and a multiparous female, with anti-mouse IgG as a secondary reagent. (**G**) Anti-KEL IgG in the serum of nulliparous or multiparous (3 pregnancies) females. (**H**) Anti-KEL IgG in the serum of females after 1,2,or 3 deliveries; 42 total pregnancies are shown; *p<0.05

Figure 5.5: Maternal anti-KEL IgG crosses the placenta and binds to KEL RBCs of pups. (**A**) RBCs from KEL positive and KEL negative pups born to mothers immunized through pregnancy was evaluated for bound anti-KEL IgG by flow cytometric crossmatching. (**B**) Serum from these same animals was evaluated for anti-KEL IgG in a direct anti-globulin test. Results are representative of 3 independent breeding experiments, n=20 pups;*p<0.05. (**C**) Anti-KEL subtypes in the serum of a representative alloimmunized mother compared to the serum of her KEL negative pups. (**D**) Newborn liver cells from a representative KEL negative and pale KEL positive pup born to an alloimmunized mother, quantitated by trucount beads (left column) or stained with anti-mouse Igs (right column). (**E**) Total number of pups and percentage of KEL positive pups was evaluated in 1st and 3rd litters of MuMT females bred with KEL males; n=8 total pregnancies; p is not significant. No anti-KEL antibodies were detected in any MuMT female; error bars indicate standard deviation.

Figure 5.6: Transfused KEL RBCs are selectively cleared in alloimmunized animals, with recipient pro-inflammatory cytokine response. (A) KEL and

wild type RBCs were labeled with DiI and DiO, respectively, prior to transfusion into females alloimmunized through pregnancy or into control recipients; (**B**) post-transfusion survival and recovery of KEL RBCs was determined by comparing a ratio of DiI KEL to DiO wild type RBCs. (**C**) RBC bound IgG was evaluated by flow cytometry 10 minutes post-transfusion. (**D**) Serum cytokine responses in alloimmunized animals, 90-120 minutes after a KEL RBC transfusion. Results are representative (A-C) or a compilation (D) of 3 experiments, n=18 animals; *p<0.05.

Figure 5.7: KEL RBC transfusions induce boostable anti-KEL alloantibodies, which result in adverse pregnancy outcomes. (A) Control animals were transfused 3 times with KEL RBCs, with anti-KEL IgG subtypes and IgM measured in pooled serum and compared to pooled serum from 12 females alloimmunized through pregnancy. (**B**) Anti-KEL IgG measured in transfusion recipients 2 weeks after each transfusion. (**C**) Naïve or transfused females were bred to KEL males, with total pups born enumerated, (**D**) percentage of pups alive at weaning evaluated, and (**E**) percentage of KEL positive pups determined. Results in (**B**) are representative of 3 independent experiments; results in (**C-E**) are a compilation of 37 pregnancies; *p<0.05.

Figure 5.1

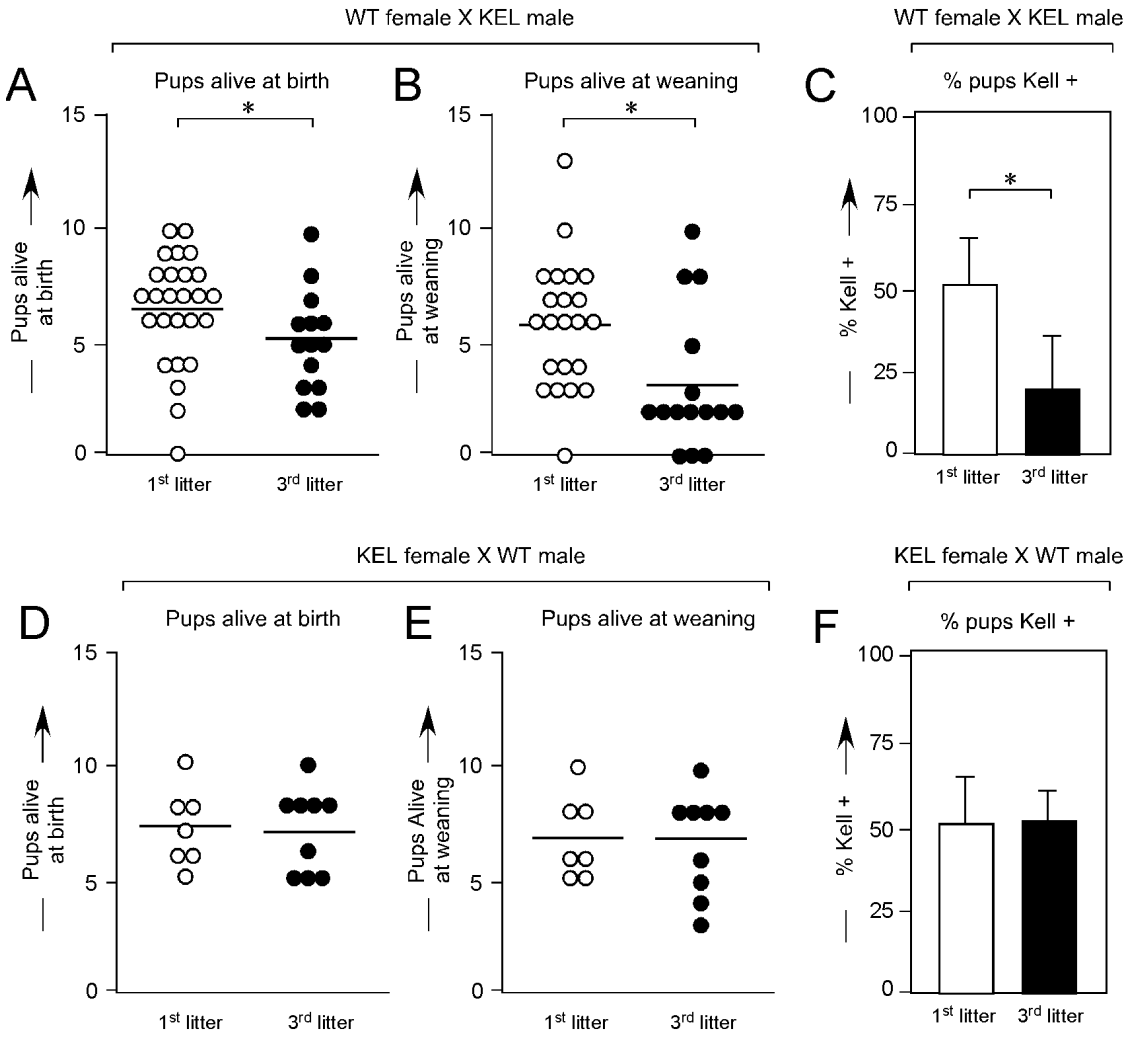

WT female X KEL male
A
Pups alive at birth
*
Pups alive at birth
15
10
5
0
1st litter
3rd litter
B
Pups alive at weaning
*
Pups alive at weaning
15
10
5
0
1st litter
3rd litter
WT female X KEL male
C
% pups Kell +
*
% Kell +
100
75
50
25
0
1st litter
3rd litter
KEL female X WT male
D
Pups alive at birth
Pups alive at birth
15
10
5
0
1st litter
3rd litter
E
Pups alive at weaning
Pups Alive at weaning
15
10
5
0
1st litter
3rd litter
KEL female X WT male
F
% pups Kell +
% Kell +
100
75
50
25
0
1st litter
3rd litter

Figure 5.2

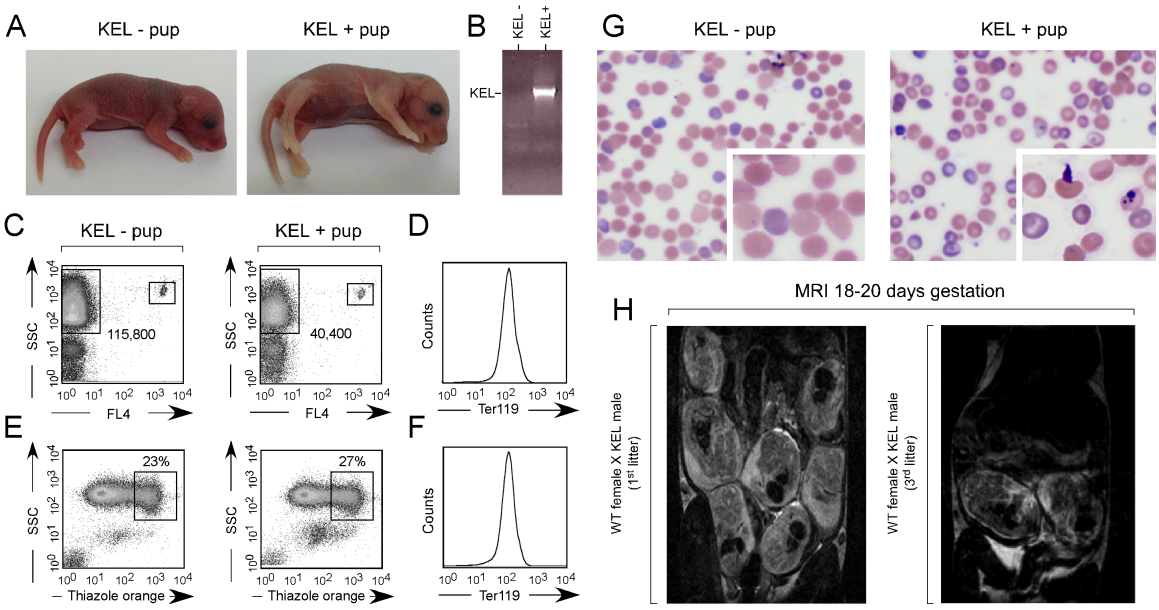

A
KEL - pup
KEL + pup
B
KEL -
KEL+
KEL-
G
KEL - pup
KEL + pup
C
KEL - pup
KEL + pup
SSC
FL4
115,800
40,400
D
Counts
Ter119
E
23%
27%
SSC
Thiazole orange
F
Counts
Ter119
H
MRI 18-20 days gestation
WT female X KEL male (1st litter)
WT female X KEL male (3rd litter)

Figure 5.3

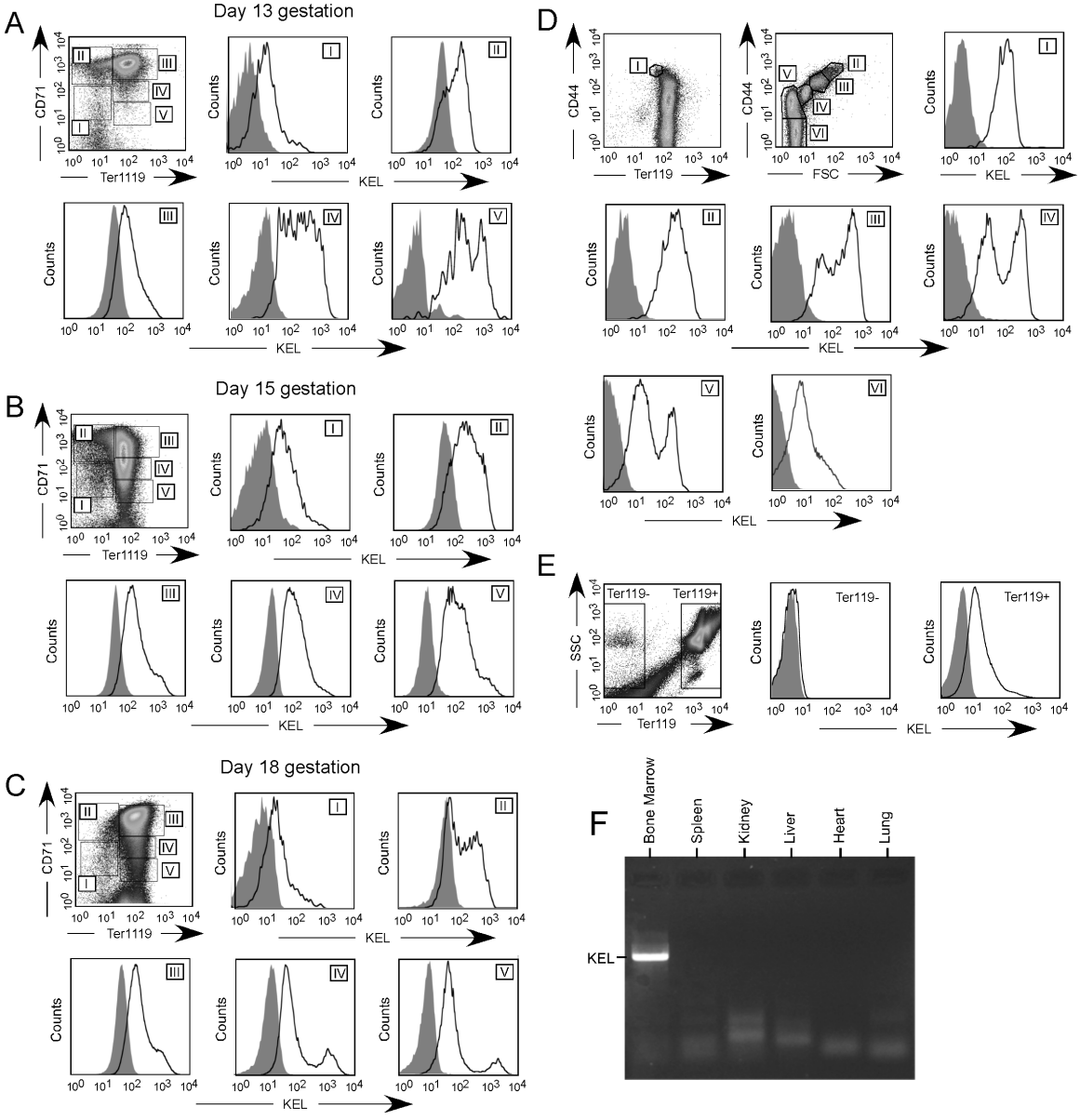

A
Day 13 gestation
CD71
Ter1119
Counts
KEL
B
Day 15 gestation
C
Day 18 gestation
D
CD44
Ter119
FSC
E
SSC
Ter119-
Ter119+
F
Bone Marrow
Spleen
Kidney
Liver
Heart
Lung
KEL

Figure 5.4

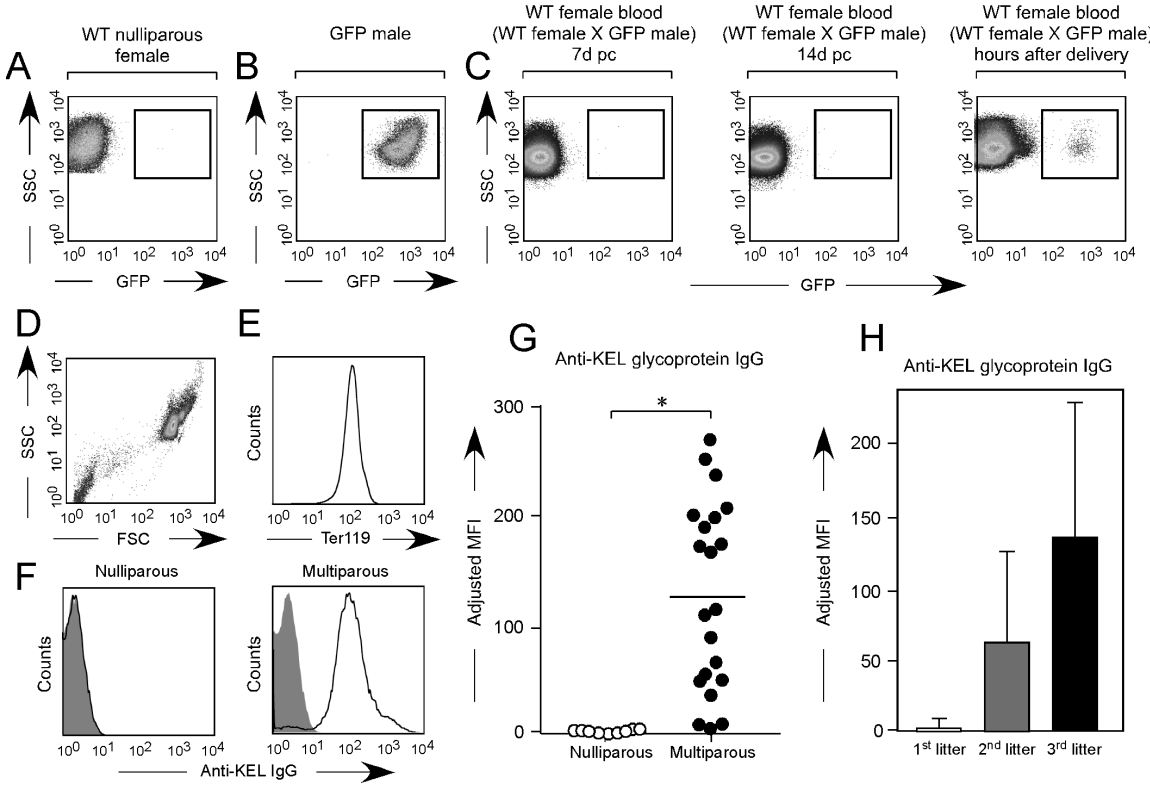

A
WT nulliparous female
B
GFP male
C
WT female blood (WT female X GFP male) 7d pc
WT female blood (WT female X GFP male) 14d pc
WT female blood (WT female X GFP male) hours after delivery
SSC
GFP
D
SSC
FSC
E
Counts
Ter119
F
Nulliparous
Multiparous
Counts
Anti-KEL IgG
G
Anti-KEL glycoprotein IgG
Adjusted MFI
*
Nulliparous
Multiparous
H
Anti-KEL glycoprotein IgG
Adjusted MFI
1st litter
2nd litter
3rd litter

Figure 5.5

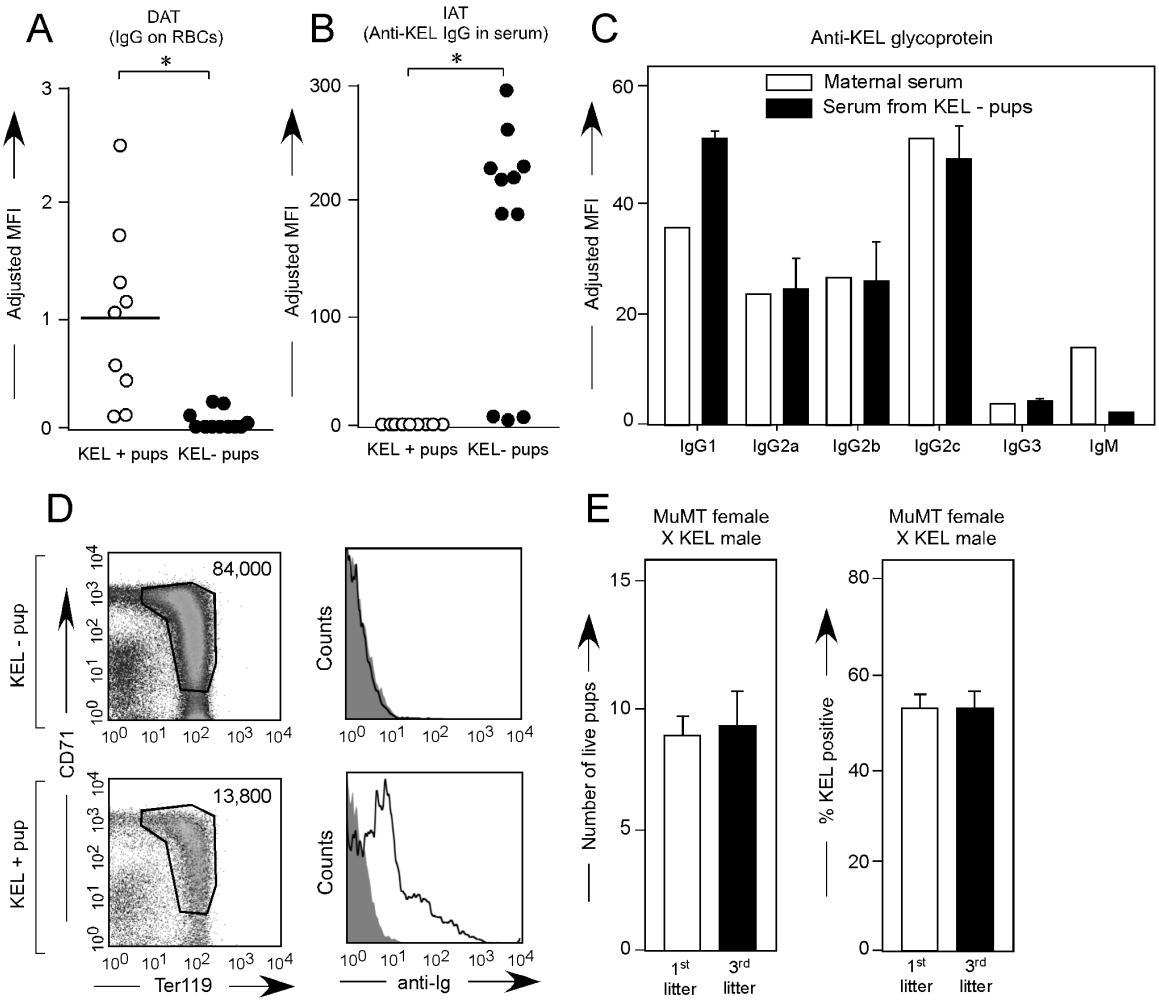

A
DAT
(IgG on RBCs)
Adjusted MFI
KEL + pups
KEL- pups
B
IAT
(Anti-KEL IgG in serum)
Adjusted MFI
KEL + pups
KEL- pups
C
Anti-KEL glycoprotein
Maternal serum
Serum from KEL - pups
Adjusted MFI
IgG1
IgG2a
IgG2b
IgG2c
IgG3
IgM
D
KEL - pup
84,000
CD71
Counts
KEL + pup
13,800
Counts
Ter119
anti-Ig
E
MuMT female
X KEL male
Number of live pups
1st litter
3rd litter
MuMT female
X KEL male
% KEL positive
1st litter
3rd litter

Figure 5.6

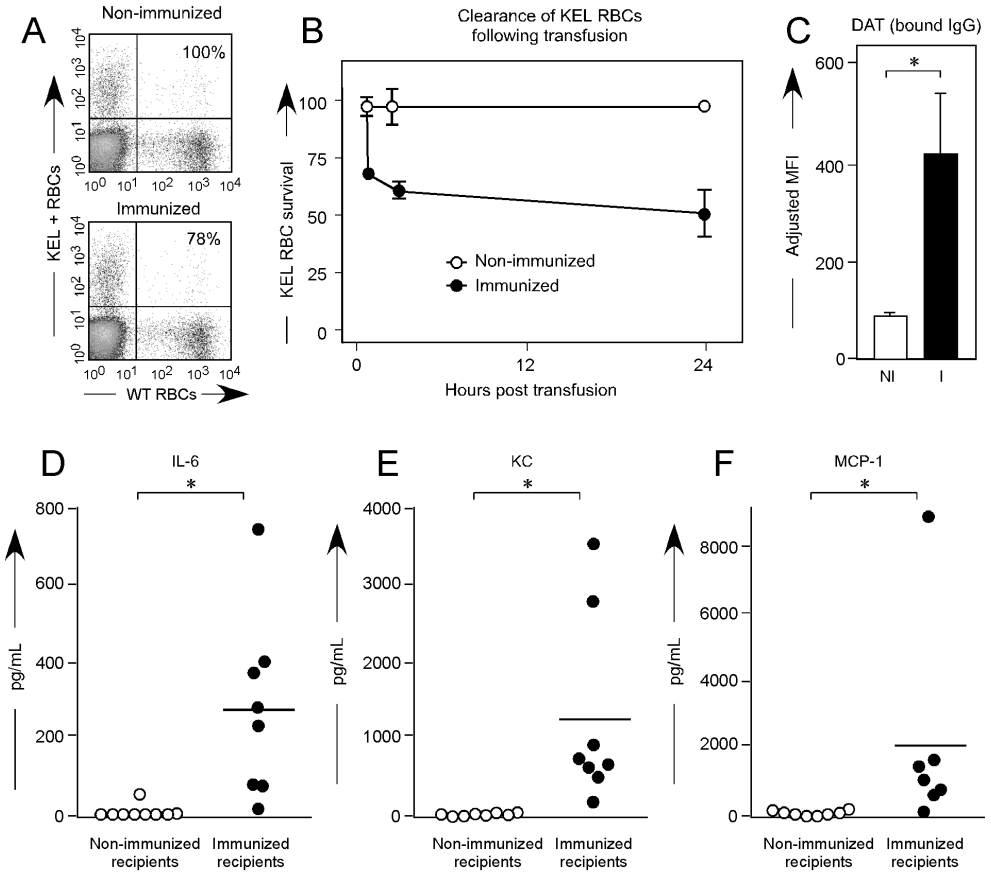
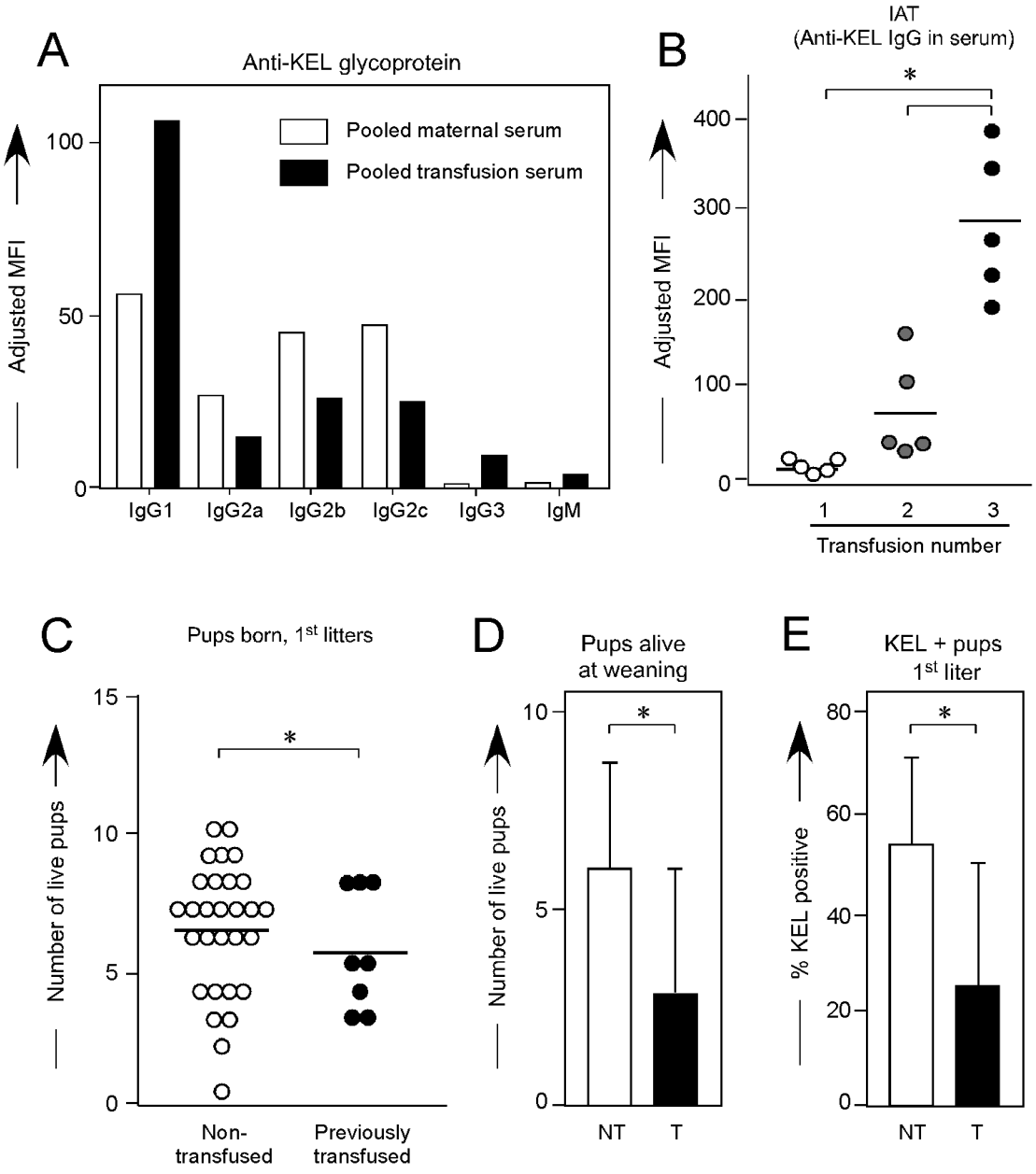

A
Non-immunized
100%
Immunized
78%
KEL + RBCs
WT RBCs
B
Clearance of KEL RBCs following transfusion
KEL RBC survival
Non-immunized
Immunized
Hours post transfusion
C
DAT (bound IgG)
Adjusted MFI
NI
I
D
IL-6
pg/mL
Non-immunized recipients
Immunized recipients
E
KC
pg/mL
Non-immunized recipients
Immunized recipients
F
MCP-1
pg/mL
Non-immunized recipients
Immunized recipients

Figure 5.7


A
Anti-KEL glycoprotein
Pooled maternal serum
Pooled transfusion serum
Adjusted MFI
100
50
0
IgG1
IgG2a
IgG2b
IgG2c
IgG3
IgM
B
IAT
(Anti-KEL IgG in serum)
*
Adjusted MFI
400
300
200
100
0
1
2
3
Transfusion number
C
Pups born, 1st litters
*
Number of live pups
15
10
5
0
Non-transfused
Previously transfused
D
Pups alive at weaning
*
Number of live pups
10
5
0
NT
T
E
KEL + pups 1st liter
*
% KEL positive
80
60
40
20
0
NT
T

Chapter 6

Discussion

SUMMARY

Blood transfusions, though often a beneficial and potentially life saving treatment for those with severe anemia, are not without risks. Historically, infections transmitted through transfused blood have been a major concern, but with the implementation of much more sophisticated and stringent screening techniques, the risks of infection have drastically decreased. Emerging evidence has indicated, however, that non-infectious serious hazards of transfusion may be associated with a significantly higher rate of adverse events associated with transfusion than previously appreciated[1, 2].

One of these risks is that the patient may become alloimmunized against blood group antigens that differ between their own and the donor red blood cells. Alloimmunization rates to red cell antigens are relatively low overall (approximately 3-5%), however in some patient groups they can be much higher, for example rates have been estimated to be as high as 47% in patients with sickle cell anemia[3]. For patients such as these, alloimmunization can represent a serious and sometimes life threatening occurrence, in fact, hemolytic transfusion reactions caused by alloimmunization to non-ABO antigens were the leading cause of transfusion-associated fatalities reported to the Food and Drug Administration last year[4]. In addition, many patients who are transfused often not only have a higher chance of generating an initial immune response to a red cell antigen, but also are at greater risk of becoming immunized against other antigens upon subsequent transfusions. In these situations, finding a compatible

unit of blood to transfuse into the patient becomes increasingly difficult, and at times impossible[5, 6].

Much is still unknown regarding the specific mechanisms of the immune response to red cell antigens, so in order to study these issues in a controlled, tractable setting our lab has generated a transgenic mouse with erythrocyte specific, stable expression of the human KEL glycoprotein, which carries one of the most clinically significant blood group antigens that has been shown to cause alloimmunization in both transfusion and pregnancy settings. [7-10].

Our initial investigations using the Kell model were to determine whether mice transfused with KEL expressing RBCs could naturally generate an immune response to the Kell antigen. We observed that naïve mice, differing only in their lack of expression of the KEL glycoprotein from the Kell transgenic mice, generate a robust, and reproducible anti-KEL antibody response when transfused with the KEL RBCs, and also occurred regardless of whether an inflammatory stimulus, such as Poly I:C, was present. Anti-KEL IgM antibodies appeared in the serum as early as 3 days post transfusion, followed soon after by the appearance of anti-KEL IgG antibodies as soon as day 7. These antibodies were shown to be capable of binding KEL clearing transfused KEL RBCs not only after a second transfusion, but also those from the first transfusion. In addition, we found that administration of the inflammatory agent Poly I:C prior to the initial or subsequent transfusion led to both higher anti-KEL antibody levels and a greater amount of KEL RBC clearance. Transfusions between mice expressing

either the Kell (K) or Cellano (k) polymorphism on the KEL glycoprotein, however, did not result in a detectable alloimmune response to either antigen[8].

Next we investigated the mechanism by which alloimmunization to the KEL RBCs occurs and discovered that the spleen, and more specifically, the Marginal Zone B-cells within the spleen were essential for anti-KEL antibody formation. Splenectomized recipients did not make detectable anti-KEL antibodies, nor did they exhibit any more clearance of the KEL RBCs than control KEL+ recipients. To specifically deplete Marginal Zone B-cells, recipient mice were treated with anti-αL (α subunit of LFA-1) and anti-α4 (α subunit of VLA-4). MZ B-cell depleted mice experienced blunted anti-KEL IgM formation, and had no detectable anti-KEL IgG response. KEL RBCs also had an increased circulatory lifespan in MZ B-cell depleted recipients as compared to isotype control antibody treated recipients. In addition, we found that although a low level of anti-KEL IgM was generated in MZ B-cell recipients, there was a reduced ability of the antibody to bind to KEL RBCs in circulation, indicating a decrease in the functional activity of the IgM antibodies. Finally, we demonstrated that subsequent transfusion in previously MZ B-depleted recipients failed to result both in antibody binding and in clearance of KEL RBCs. Taken together, these data reveal an essential role for MZ B-cells in the generation of functional anti-KEL alloantibodies and suggest a possible therapeutic target for prevention of alloimmune responses to red cell antigens in the future.

In humans, incompatible transfusion of Kell+ RBCs into an immunized, Kell- recipient can cause severe hemolytic transfusion reactions, which is also

reflected in our murine model. In a WT recipient immunized against KEL, transfusion of KEL RBCs induces rapid clearance as well as other symptoms characteristic of a hemolytic transfusion reaction, including the induction of a pro-inflammatory cytokine storm, as well as the presence of free heme in both the serum and urine of immunized recipients following transfusion, indicating hemolysis of the transfused RBCs. Using recipients that lacked either the common gamma chain or the complement protein C3, we discovered that both Fcγ receptors and complement participate in clearance. In the human setting, incompatible Kell RBCs can sometimes modulate their antigen in order to escape detection from the immune system and avoid destruction. Similarly, using our model we noted an initial period of rapid destruction of transfused KEL RBCs followed by a period of much slower clearance, indicating the possible presence of a resistant population of KEL RBCs. We analyzed the levels of detectable KEL glycoprotein as well as anti-KEL antibody bound to the transfused cells following transfusion, and observed that both levels experienced a significant rapid decrease in immunized WT as well as FcγR KO recipients. A slight decrease was also noted in immunized C3KO recipients, but to a much lesser degree and with much slower kinetics. We also determined the levels of C3 bound to the transfused RBCs and found that although C3 rapidly binds to anti-KEL antibody coated RBCs, it is rapidly converted into its inactive form. We hypothesize that this inactive C3 is masking the antibody-coated KEL RBCs and protecting the transfused cells from further immune-mediated destruction [10].

Kell is not only a clinically significant RBC antigen in the setting of transfusion, but also in pregnancy where it has been shown to lead to hemolytic disease of the fetus and newborn. Little is known regarding the mechanism of the immune response to red cell antigens during pregnancy, and historically there has not been a model in which the induction and consequences of alloimmunization to fetal red cells can be studied. First we determined the level of KEL glycoprotein expression on red cell precursors during development by analyzing KEL expression in the fetal liver and bone marrow from day 13 post-conception through delivery. KEL expression was detectable on all RBC precursors in the fetal liver and bone marrow but not in any non-hematopoietic tissues tested, indicating similar expression patterns to the human KEL glycoprotein during erythropoiesis. When KEL+ males were mated with WT (KEL-) females, we observed a decrease in overall number of pups born, number of pups alive at weaning, and the percentage of surviving pups that were positive for expression of the KEL glycoprotein with each successive pregnancy. Anti-KEL IgG antibodies were detectable in the mothers following delivery, and an increase in antibody level was observed with each additional pregnancy. In contrast, when the female was KEL+ and the male KEL-, no such decreases were observed and no anti-KEL antibodies detected. MRIs of pregnant WT females pregnant for the third time from a KEL+ male showed smaller litters in utero compared to the first pregnancy, and the KEL+ pups that were delivered in the third litter or later exhibited classic symptoms of hemolytic disease of the fetus and newborn, including anemia, fetal hydrops, reticulocytosis, and

decreased survival. Antibodies were found to be the likely cause of this observed pathology in the pups, as MuMT mothers who are incapable of producing antibody had no change in litter size and did not deliver pups with symptoms of HDFN. Furthermore, we showed that antibodies generated in response to KEL through either transfusion or pregnancies are clinically significant in both settings[9].

DISCUSSION

Red blood cells are unique as cells and immunogens

Red blood cells are a unique type of cell with the primary responsibility of delivering oxygen to tissues and organs around the body. Mature erythrocytes lack a nucleus and mitochrondria, and no longer have the ability to synthesize protein. In a normal, nucleated cell, proteins and other molecules expressed in the membrane can be internalized and re-expressed, allowing for changes in expression levels throughout the lifetime of the cell. Conversely, in a red cell the molecules expressed on the cell surface at the time it develops from a reticulocyte to a mature erythrocyte are the only surface molecules that it will ever express throughout its 120 day life span, any changes or alterations that occur to these molecules are permanent. In some cases, modifications to these cell surface proteins or other membrane components can result in morphological changes to the red cell, and since these cells rely heavily upon their deformability in order to navigate through the microvasculature, this can shorten their survival rate[11].

Due to their significant differences in characteristics from normal cells, red cells are a unique type of immunogen as well. Though their expression level is static, many cell surface proteins are expressed on the red cell membrane, with hundreds of polymorphic epitopes that can differ from person to person. Although an individual may be exposed to several foreign blood group antigens

when given a blood transfusion, alloimmunization is a relatively rare event, only occurring in approximately 3-5% of transfusions[6]. There are several potential reasons for this, including that all red cell units should be without microbial contamination prior to transfusion and have also been leukoreduced, removing possible sources of inflammation or danger signals such as the toll like receptor ligands that may be present on pathogenic microbes[12]. In addition, transfused red cells are contained within the vasculature, and this intravenous delivery of antigen has been described to more often promote tolerance than other routes of exposure, especially in the absence of danger signals. This may be because red cell antigens are only typically seen by immune cells in the spleen and liver, unlike foreign antigen in the tissue which drain into local lymph nodes[13, 14].

Additionally, whereas the entirety of a pathogenic microbe may be recognized as foreign by the human immune system, red cell antigens generally only differ by one amino acid between the donor and recipient. This small change may not activate the immune system if it does not create a novel B or T cell epitope, or if it cannot be expressed within the antigen binding pocket of MHC class II molecules on antigen presenting cells. Furthermore, the lack of expression of MHC- class I molecules on human erythrocytes protects them from the rapid and robust rejection which occurs in the case of transplantation or transfer of any other type of cell, tissue, or organ transfer between donor and recipient[15, 16].

Therefore, although many model systems have been developed to study various antigens such as those on microbes or transplanted tissues, red cell

antigens have distinct characteristics that necessitate the requirement for a unique model system. That being said, a red cell model may also be useful in studying other types of antigens, specifically in determining the consequences of antibody and/or complement engagement, since the antigen cannot be internalized or re-expressed and thus any antibody induced modulation to the antigen would be observable. Also, control and antigen positive red cell populations can be compared using our lipophilic dye labeling strategy and can be easily tracked as they are confined within the vasculature. Thus, the development of novel red cell model systems may have implications not only for transfusion medicine, but also for the study of antigens in other systems as well.

The use of transgenic mouse models to study red cell alloimmunization

As discussed in Chapter 2, alloimmunization to red cell antigens is a clinically significant problem that can cause severe adverse events following transfusion, such as acute or delayed hemolytic transfusion reactions, or potentially fatal complications during pregnancy, such as hemolytic disease of the fetus and newborn. Much is still unknown about the pathophysiology of these disorders, and the mechanisms by which they occur. Being able to study these immune responses in humans would be ideal, however due to obvious ethical limitations, human studies often are only based on retrospective observations, and are inherently confounded by a number of unknown variables making it difficult to

draw any definite conclusions. Using animal models provides the opportunity to perform controlled studies using genetically defined research subjects. Specifically, inbred mouse strains are a particularly good model for human biology, as mice are quite similar to humans genetically; only approximately 300 genes differ between the two species and nearly 99% of human genes have a mouse homolog[17].

The immune systems of mice and humans are quite similar as well, but there are some general differences that may affect the study of transfusion reactions. For example, human blood is more neutrophil rich, and mouse blood is more lymphocyte rich[18]. It is unknown so far whether this difference in lymphocyte/neutrophil balance has any effect on red cell alloimmunization. What could be of more importance to our studies of red cell clearance mechanisms are the differences between mouse and human FcγR and IgG subtype expression. Humans have two IgG receptors, FcγRIIA and FCγRIIC that are not found in mice[19]. In addition, mouse and human IgG subtypes are not exact homologs of each other, and the recipient mouse strain used in our studies, C57BL/6, has no expression of one subtype of IgG, IgG2a[20]. Although different IgG subtypes can vary in their ability to bind Fcγ receptors, and in theory this may have an impact on immunological processes such as antibody-mediated cell clearance, the differences in IgG and FcγR interactions between human and mice are generally not considered significant[21].

One additional difference between mice and humans that may have implications for the study of the immune response to red cell or other blood borne

antigens is that the structure of the spleen differs slightly between species. Of particular importance to our research is that the structure of the marginal zone seems to be slightly more sophisticated in humans than in mice, containing an inner and outer marginal zone surrounded by a perifollicular area. Because of this structural difference, lymphocyte entry into the spleen is slightly different between humans and mice, however it is not thought to have any affect on the entry of red cells into the spleen[22].

Despite these and other small differences that exist between the immune systems of mice and humans, their biology in general is quite similar, making mice the most widely used species for modeling human disease and an ideal model for the study of the immune response to red cell antigens. Many murine models currently exist for the study of various aspects of the immune responses to pathogens or transplanted tissue, however since blood group antigens are fundamentally distinct from other types of antigens in their level of antigenic difference, route of exposure, and the cell type they are expressed upon, they require a unique model system. Because of these specific characteristics, animal models of red cell antigens have been difficult to generate and many have failed to replicate a similar immune response to humans. These models have been useful in enhancing the understanding of certain elements of red cell alloimmunization and clearance, however in each there are aspects, which make their biology considerably different from what occurs in the human system[23-30].

Our novel Kell transgenic model is, to our knowledge, the first with the ability to fully recapitulate the characteristics of the human response to a clinically

significant red cell antigen. Use of this model may provide valuable information regarding both the induction of the immune response as well as the fate of incompatible red cells in an immunized recipient. Ultimately, information gained from the use of this model may improve the ability of physicians to better predict when alloimmunization to antigens on transfused red cells will occur or could potentially lead to the discovery of a therapeutic target for the prevention of alloimmunization.

The role of the spleen in the generation of the immune response to RBC antigens

In order to investigate the potential mechanisms involved in alloimmunization, it is important to identify the location in which red cell antigens are recognized by the immune system. In contrast to foreign antigens from non-blood borne pathogens or transplanted organs which drain into the lymphatics and are processed in local lymph nodes, red cell antigens are confined to the vasculature and as such, their only opportunities for detection by the immune system are through exposure within the spleen or liver.

The liver contains many immunologically active types of cells, including liver-specific macrophages known as Kupffer cells, DCs, and various lymphocytes. Although these cells are in some situations capable of generating a pro-inflammatory response, the liver is usually a tolerogenic organ. This suppressive environment is necessary because the liver is not only exposed to

cells within normal circulation, but also receives blood from the portal vein, which contains antigens and microbial products from digestion and from the intestines. As such, innate cells within the liver down-modulate costimulatory molecules and upregulate IL-10, and T cells are often induced to become T_{regs}. This suppressive environment can only usually be reversed by very strong pathogen specific signals, and since RBCs are generally a weak immunogen, the liver is an unlikely location for the generation of the immune response against them[31]. It is therefore reasonable to hypothesize that the spleen must be involved in RBC alloimmunization.

The idea that the spleen is involved in alloimmunization to red blood cells is not a new one, in fact splenectomy has long been used as a treatment for autoimmune hemolytic anemia (AIHA) [32]. Studies performed over 40 years ago demonstrated that antibody coated RBCs are removed almost exclusively by the spleen [33]. More recent murine studies have also described the essential role of the spleen in alloimmunization to a model antigen, mHEL [34]. What has not yet been elucidated, however, is the precise mechanism by which the immune response is initiated within the spleen and which specific cell types are involved.

The spleen contains many conventional immune cells, such as B-cells, T-cells, macrophages, and dendritic cells, but also contains some unique cell types such as marginal zone macrophages, marginal zone metallophilic macrophages, and marginal zone B-cells. These cells are the first to come into contact with cells within the blood as they enter the spleen from the circulation, and MZ B-

cells have long been recognized for their ability to mount rapid IgM responses to blood borne pathogens [35]. Marginal zone B-cells have also been recognized for their ability to migrate to the white pulp where they and act as APCs, activating CD4+ T-cells [36]. Furthermore, it has also more recently been discovered that MZ B-cells themselves have the ability to undergo class switching and hypermutation to produce antigen-specific IgG responses to a bacteria within the blood [37-41]. Though performed using a bacterial pathogen, these studies suggested that marginal zone B-cells may also play a role in the immune response to red cell antigens as well.

The studies that we have performed comparing alloimmunization to transfused KEL RBCs in WT vs MZ B-cell depleted mice support the hypothesis that MZ B-cells play a role the ability of a recipient to generate an IgG response. We have not yet confirmed whether MZ B-cells are themselves directly responsible for the generation of IgG alloantibodies, or whether they somehow are playing an essential role in the conventional B-cell response, such as by shuttling antigen to the white pulp of the spleen and activating CD4+ T cells. Regardless, these data indicate that MZ B cells are not only involved in the initial immune response, but also play an essential and previously unrecognized role in the general humoral immune response to the Kell antigen, and possibly other red cell antigens and blood borne pathogens as well.

Taken together, these data provide the first example of the prevention of RBC alloimmunization as a result of a targeted treatment and may lay the groundwork for the development of a prophylactic therapeutic with the ability to

prevent alloimmunization to transfused red cell antigens. For patients who require chronic transfusions, a therapeutic such as this could profoundly improve the treatment of disease by enhancing the therapeutic capacity of transfusion while increasing the availability of compatible RBCs and preventing hemolytic transfusion reactions.

It is important to keep in mind, however, that some patients with sickle cell disease auto-infarct their spleens, which is thought to render them functionless, however they are still capable of generating alloimmune responses to transfused red cell antigens. This contrasting evidence seems to argue against the role of the spleen in alloimmunization, or may suggest an alternate mechanism for immunization in these patients. Further research investigating the mechanisms of alloimmunization as well as the true functional capacity of an infarcted spleen will be required to clarify this apparent contradiction [42].

Mechanisms of clearance or survival of transfused RBCs

Transfusion of crossmatch incompatible blood often results in the immune-mediated clearance of the incompatible RBCs, which can have serious and sometimes fatal complications. However, antibody-mediated destruction is not the inevitable outcome when incompatible RBCs are transfused into an immunized recipient. In some cases, cells continue to circulate normally despite being bound by antibody. Alternatively, cells can sometimes undergo changes in

which the target antigen is altered or removed in some way in order to protect the cells from immune destruction[23, 43, 44].

It is often impossible to predict which process will occur prior to transfusion of the incompatible cells, and symptoms in the patient can vary widely depending on the mechanism or degree of red cell destruction, therefore in order to develop potential therapies or procedures to minimize or prevent these responses, we must better understand both the specific mechanisms of immune-mediated clearance as well as mechanisms involved in how cells avoid destruction.

It is widely known that in humans, antibody induced clearance of red cells following an incompatible transfusion can occur intravascularly, by a complement-mediated mechanism, or extravascularly, involving Fcγ receptors on phagocytes[45-47]. Additional mechanisms of immune mediated destruction have also been suggested, such as eryptosis induced by Ca^{2+} influx, destruction by scavenger receptor expressing phagocytes due to phosphatidyl serine expression on the red cell membrane, or sequestration of agglutinated antibody bound red cells leading to destruction [30, 48, 49].

Murine models of RBC clearance have provided valuable information regarding Fcγ mediated and agglutination mediated destruction of RBCs [30, 50]. Additional studies using murine model systems have enhanced our understanding of certain characteristics of antigens and antibodies that may play a role in antigen modulation, such as requirements for the quantity of antibody bound or density of antigen on the red cell[51-53].

Our Kell transgenic mouse is the first model to demonstrate the involvement of complement component C3 in clearance as well as antigen modulation. Besides providing mechanistic information regarding these processes, these findings also suggest the potential use of complement as a therapeutic strategy, whereby incompatible RBCs can be transfused into an immunized patient by pre-treating the red cells with antibody and complement in order to mask the antigen from the recipient immune system.

Developing a strategy for induced antigen modulation may also help to treat a variety of autoimmune disorders, not just those associated with red cell antigens. In addition, understanding the mechanisms underlying antigen modulation may lead to the development of treatments with the ability to prevent the phenomenon from occurring in situations in which immune mediated destruction is preferred, such as in cancer cells which can sometimes downregulate their target antigen. Therefore, the contribution of the Kell model in elucidating the mechanisms of antigen modulation may have implications not only in transfusion medicine but in understanding the pathophysiology of other diseases and developing treatments as well.

Implications of an animal model for hemolytic disease of the fetus and newborn

Chapter 5 describes our studies utilizing our Kell transgenic mouse as a model for hemolytic disease of the fetus and newborn (HDFN), a condition that

has severe consequences for the fetus, with symptoms including anemia, hyperbilirubinemia, erythroblastosis fetalis, and in some cases, death[54, 55]. By mating KEL- females with KEL+ males, we demonstrated that paternally derived KEL expressed on fetal RBCs leads to the development of maternal alloantibodies, which are boostable upon subsequent pregnancies or by transfusion. These alloantibodies adversely affected developing fetuses, which displayed symptoms characteristic of HDFN and had reduced survival.

Prior to the description of this model, no animal models existed that could replicate pregnancy-induced alloimmunization resulting in HDFN. A rabbit model generated in the 1990's was able to demonstrate maternal antibody binding to fetal RBCs resulting in symptoms of HDFN in pups, however the induction of the alloimmune response in the mothers was not induced naturally during pregnancy but required several immunizations of antigen combined with adjuvant in order to occur[56, 57]. Many attempts have been made to generate a transgenic mouse with RBC expression of the human RhD antigen in order to study HDFN, however none have so far been successful. Thus, our model represents the first animal model of HDFN with the ability to recapitulate many key features of the human disease.

The development of this model is an important step in potentially discovering and/or testing new therapeutics for the prevention or treatment of HDFN. As of now, the only therapy available to prevent HDFN is the administration of polyclonal anti-D (RhoGAM) to RhD negative mothers pregnant by an RhD father. RhoGAM has been very successful in preventing RhD

associated HDFN, and since its introduction in 1968 the rate of RhD associated pregnancy alloimmunization has decreased by 95%. However, this treatment only prevents HDFN caused specifically by RhD mismatch, yet there are over 50 other antigens that have been identified to be associated with HDFN, for which there is no treatment. In addition, the mechanism of action of RhoGAM remains largely unknown, inhibiting the development of additional prophylactic therapeutics[58]. Furthermore, the only treatment available for a developing fetus with HDFN is intrauterine transfusion, which in itself is dangerous and may cause fetal death[59, 60]. Our Kell model may provide valuable insight into the mechanisms of both the disease as well as the only known prophylactic therapy.

Limitations of the Kell transgenic model

Similar to all models, there are some caveats that must be considered when using a mouse model to study human disease. Although the anatomy and physiology of the mouse and human immune systems are similar, they are not exactly the same and therefore caution must always be used when extrapolating findings acquired from mouse studies to the understanding of human biology.

Besides the general differences between the mouse and human immune systems, there are some differences specific to our system that must be kept in mind. To generate the Kell transgenic mouse, the Kell transgene is randomly inserted into the genome. It has been verified that this has not resulted in any

alteration to the morphology, circulatory life span, or behavior of the KEL RBCs, but there may be other consequences that are as of yet unknown[7].

Another limitation of the Kell transgenic mouse system is that it does not exactly model either the human Kell or the RhD systems, but instead displays some features of each. It resembles RhD in that the entire glycoprotein is either present or absent from the red cell membrane, instead of the human Kell scenario where only a single amino acid is changed which creates a distinct epitope. Also, unlike in humans, a transfusion of transgenic red cells expressing the antithetical antigen KEL1 (Kell) into recipients expressing the KEL2 (cellano) antigen or vice versa does not result in anti-KEL1 or anti-KEL2 antibodies. Only when the recipient lacks the entire human KEL glycoprotein does it make a response to KEL RBCs. The clinical symptoms of Kell alloimmunization and the characteristics of the alloantibody, however, are more similar to the human Kell than the RhD system.

CONCLUSIONS

In sum, this dissertation has described the use of a novel murine model with RBC specific expression of the human KEL glycoprotein, one of the most clinically significant blood group antigens, in the study of both transfusion and pregnancy induced alloimmunization. This model is the first to recapitulate the fundamental features of the human immune response in both situations, thus

providing a system in which specific mechanistic details of both the induction and consequences of the alloimmune response to red cell antigens can be studied. Already we have identified a specific cell type, namely the marginal zone B-cells, which are required for the generation of functional alloantibodies against the KEL glycoprotein on the transfused RBCs, and have shown that a targeted but temporary depletion of these cells can prevent alloantibody formation and a hemolytic transfusion reaction. In addition, we have discovered a potential mechanism by which transfused cells can become protected against immune-mediated destruction, suggesting that it may be possible to manipulate this strategy to use as a possible treatment to prevent alloimmunization to or destruction of transfused RBCs. Furthermore, we have demonstrated the utility of the Kell transgenic mice not only in modeling transfusion reactions, but also as a model for pregnancy induced allimmunization and hemolytic disease of the fetus and newborn. Although there are obviously differences between human and mouse biology, this model is the closest system by which we can study these immune responses in a controlled, tractable manner. Ultimately, the goals for the use of this model system are to decrease the dangers associated with transfusion or pregnancy induced alloimmunization to red cell antigens, as well as to suggest and/or test novel therapeutic targets for the prevention or treatment of the conditions and diseases associated with red cell alloimmunization.

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