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The consequences of antibody binding to red blood cells in alloantibody responses
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The consequences of antibody binding to red blood cells in alloantibody responses

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Abstract

The consequences of antibody binding to red blood cells in alloantibody responses
By Amanda Mener

Red blood cell (RBC) transfusions provide a life-saving intervention in the setting of hematologic conditions and trauma. However, it is not without risk. Repeated RBC transfusions can lead to formation of alloantibodies against allogeneically distinct antigens on the surface of transfused RBCs. These RBC alloantibodies increase the risk of hemolytic transfusion reactions, or in the case of pregnancy, hemolytic disease of the fetus and newborn, as well as causes difficulty in finding compatible RBCs for future transfusions. The mechanism(s) of how RBC alloantigens induce alloantibodies and furthermore, the immunologic consequences of antibody binding to RBCs following transfusion of RBCs, remain poorly understood. Currently, there is only one pharmacologic agent available to actively prevent the development of RBC alloantibodies, Rh immune globulin (RhIg), which consists of pooled plasma from donors purposely exposed to RhD^+ RBCs. The ability of passive administration of antibodies to prevent the *de novo* development of alloantibodies is termed antibody-mediated immunosuppression (AMIS). However, the mechanism of how AMIS occurs is not well-understood. Furthermore, with the decreasing availability of plasma donors and the ethical limitations in purposefully alloimmunizing patients against RBC alloantigens other than RhD, there is a critical need to better understand the mechanism(s) of AMIS in order to develop alternatives to RhIg. Given previous studies suggesting that decreases in the level of surface antigen, termed antigen modulation, may account for one mechanism of AMIS, we utilized a murine model of RBC alloimmunization, in which RBCs express a fusion protein consisting of hen egg lysozyme (HEL), ovalbumin (OVA) and human Duffy (HOD RBCs). Through exposure of mice to HOD RBCs in the presence or absence of anti-HEL monoclonal antibodies, we explore how monoclonal antibody-induced changes in the level of the target RBC antigen impacts the development of an anti-HOD alloantibody response. Additionally, through utilizing RBCs that express both the clinically-relevant HOD and KEL RBC antigens on the same RBC, we also investigate whether AMIS is antigen-specific and whether this antigen-specific AMIS occurs through antigen modulation.

Furthermore, as RhD and HOD RBCs do not induce complement fixation and complement has been shown to be important in the development of antibody responses, we next sought to determine how complement impacts the development of RBC alloantibodies utilizing a model of RBC alloimmunization that induces complement fixation, the KEL RBC antigen. Unlike previous studies describing complement as an adjuvant in the development of an antibody response, we actually observe that mice deficient in complement component 3 (C3 KO) exhibited increased alloantibody responses against the KEL antigen due to a decrease in antibody-induced antigenic changes on the RBC surface, indicating that complement negatively regulates the development of an antibody response to the KEL RBC antigen.

Finally, given the differences in IgG subclass development observed between C3-deficient and wild type mice in response to KEL RBC transfusion and the involvement of $CD4^+$ T cells in regulating class switching, we explore the role of $CD4^+$ T cells in the

development of the alloantibody response to KEL. We find that in the presence of C3, mice develop alloantibody responses to KEL even in the pharmacologic or genetic absence of $CD4^+$ T cells. However, C3 KO and mice deficient in complement receptors 1 and 2 are completely dependent on $CD4^+$ T cells to develop an anti-KEL IgG response, suggesting that C3 fixation and interaction with complement receptors regulates whether the antibody response to KEL is T-independent or T-dependent.

Taken together, these findings suggest that the consequences of antibody binding to the RBC surface, acquired either through passive administration of antibody, in the case of AMIS, or following *de novo* antibody development, include both complement-independent and -dependent antigen modulation. This antigen modulation impacts not only the subsequent development of an alloantibody response to RBC antigens, but also the immunologic pathways involved. These findings contribute to our understanding regarding how the mechanism of alloantibody development to RBC antigens may vary by the individual antigen.

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Chapter 1: Introduction

A BRIEF HISTORY OF TRANSFUSION MEDICINE

Long before medical professionals discovered that red blood cells (RBCs) could be transfused from one person to another as a life-saving intervention, people have been fascinated with blood's properties. For example, the Roman author Pliny the Elder and Greek philosopher Celsus both described how people rushed into stadiums following battles between gladiators to drink the blood of dying gladiators, believing that the weak could gain strength by drinking the blood of the strong (1). This fascination with blood continued as Galen, a Greek physician, believed that the four humors phlegm, blood, yellow bile and black bile constituted bodily fluids that when unbalanced, contributed to disease. The belief in the four humors led to the practice of bloodletting as a cure for a multitude of diseases—a practice that spanned centuries from Hippocrates to the King of England Henry VIII and George Washington. However, the actual practice of transfusing blood from one individual into another seemed inconceivable until William Harvey described the organization of the circulatory system in 1616, which was later published in 1628 (2). Because Harvey pumped water into the circulation of a dead man, rather than blood, he is not credited with having described transfusion of blood (3). In fact, the originator of the concept of blood transfusions is believed to be Francesco Folli of Florence, who published the first book on transfusion, where he described an apparatus required for transfusion, though he admits at the end of his book to never having actually performed a transfusion (4). Later, in 1666, Richard Lower described the first direct transfusion from the carotid artery of one dog into the jugular vein of another. However, many of his transfusions failed because of clotting within the tubes, as anticoagulants were not yet discovered (5). Experiments involving transfusion between animals

progressed to transfusing blood from animal to human, when in 1667, Jean Baptiste Denis transfused lamb blood into a 15 year-old boy in Paris, whose symptoms of extreme exhaustion may have indicated profound anemia. Although Denis transfused two recipients with animal blood without incident, the third and fourth recipients died. Given the deaths of Denis' transfusion recipients, experiments with transfusions declined and within 10 years, it was prohibited in both England and France (6). Transfusion as a technique remained dormant for 150 years, until James Blundell, an obstetrician, described experiments that involved transfusing dogs using syringes. He was the first to state clearly that only human blood should be used for human transfusions. The first documented transfusion with human blood took place in 1818 when a man, with obstruction of his pylorus due to a carcinoma, was transfused with human blood over 30 to 40 minutes. In total, Blundell performed ten transfusions, five of which were successful, with most of his successful transfusions used to treat postpartum hemorrhage (6, 7).

Despite the fact that Blundell provided evidence against the use of animal blood in humans, the actual technical problems associated with transfusion were not resolved, such as how to physically remove blood from one individual and transfuse it into another individual. While Blundell described the use of a "gravitator," where blood from the donor flowed down a funnel-like device and then from a flexible cannula into the patient's vein, without anticoagulants, only a portion of the donated blood reached the recipient. Additionally, without compatibility testing, although hemolytic transfusion reactions were not recognized, they most likely contributed to the deaths associated with early transfusions. In fact, it is estimated that because many of the donors and recipients

in early transfusions were Caucasian, even without matching for donor or recipient, by chance, 64.4% of transfusions were theoretically compatible (8).

Karl Landsteiner revolutionized transfusion medicine in 1900 when he observed that the sera of some individuals agglutinated with RBCs of others (9, 10), naming the blood groups A, B and C (now known as O). For his novel discovery of RBC antigens, which would impact not only transfusion medicine, but also create a foundation for transplantation as well, Landsteiner was awarded the Nobel Prize in Medicine in 1930. Shortly thereafter, von Decastello and Sturli described a fourth blood group antigen, AB (11). However, even with Landsteiner's critical observations regarding the major blood group antigens, matching as a practice was not implemented until Ludvig Hektoen of Chicago stated that donor and recipient should belong to the same blood group antigens prior to transfusion (12). Later, Reuben Ottenberg studied isoagglutination (13) and published a series on pre-transfusion testing with 128 cases (14). Despite these advances in pre-transfusion donor and recipient matching, transfer of blood from one individual to another was finally facilitated when the surgeon Alexis Carrell developed a technique of end-to-end vascular anastomoses to bring the ends of vessels into close apposition (6). The development of Unger's two-syringe, four-way stopcock further facilitated direct transfusion and ended the practice of arteriovenous anastomoses as a method of transfusion (6).

Despite the discovery of the major blood group antigens and the apparatuses that made transfusion from one individual to another possible, the absence of anticoagulation forced the process of blood collection to remain tied to blood transfusion. However, with the knowledge gained during World War II that sodium citrate and acid-citrate-dextrose

solutions could be utilized as anticoagulants, the donation process could be separated from the actual transfusion. Furthermore, with the establishment of the first blood bank by Bernard Fantus at Cook County Hospital in Chicago, blood was stored under refrigeration then warmed prior to transfusion (15). Thus, over the course of many centuries, blood evolved from a mythical humor that could give strength to those who consumed it to a life-saving intervention that could be properly typed and stored prior to transfusion.

However, the transfusion of RBCs is not without consequence. In addition to A, B, and O, there are hundreds of other antigens on the RBC surface. Chronic RBC transfusions or pregnancy can lead to the development of alloantibodies against these RBC alloantigens. These RBC alloantibodies can potentially cause hemolytic transfusion reactions or cross the placenta, leading to hemolytic disease of the fetus and newborn. The only current prophylaxis to actively prevent the development of Rh alloantibodies is Rh immune globulin (RhIg) specifically against the RhD RBC antigen. This ability of antibodies to prevent the development of a downstream alloantibody response is known as antibody-mediated immunosuppression (AMIS); however, the exact mechanism governing AMIS is still not well-understood. Furthermore, given the paucity of RhIg donors and the ethical limitations with introducing antigen-positive RBCs to antigen-negative donors to generate anti-alloantigen antibodies against other RBC antigens, there is a critical need to not only develop monoclonal alternatives to RhIg, but also develop therapies to prevent the development of antibodies against other RBC alloantigens. Therefore, in this thesis, we explore the consequences of antibody binding to RBCs, including, but not limited to AMIS. We utilize two RBC model antigens to accomplish

this: murine RBCs that transgenically express a fusion protein of hen egg lysozyme (HEL), ovalbumin (OVA) and human Duffy (HOD), as well as RBCs that express human KEL. We investigate the ability of monoclonal antibodies to induce AMIS through antibody-induced antigen changes of HOD RBCs. Additionally, as antibodies against RhD and HOD RBCs do not induce complement fixation post-transfusion and complement has been described to be important to the development of antibodies against various viruses and bacteria, we utilized a model of the KEL RBC antigen that induces complement fixation post-transfusion to explore how complement is involved in the adaptive immune response against RBC antigens. Therefore, this introduction first discusses current hypotheses regarding how AMIS occurs, followed by a discussion of the complement system's role in regulating the adaptive immune system.

ANTIBODY-MEDIATED IMMUNOSUPPRESSION

In addition to the ABO major blood group antigens, there are hundreds of other polymorphic antigens on the RBC surface, of which the RhD, KEL and Duffy antigens are the most pertinent to the studies discussed in this dissertation. While hemolytic transfusion reactions caused by the ABO blood group antigen barrier can be avoided simply by matching donors according to ABO blood type, the same strategy does not necessarily apply in the case of some minor blood group antigens, where some patients with hemaglobinopathies are chronically exposed to transfused blood products, but current transfusion medicine practices may only match donor and recipient for a limited number of 300 potential RBC alloantigen differences. Furthermore, in the case of pregnancy when the fetus and mother differ in their expression of minor RBC antigens,

there are currently no methods to prevent the development of RBC alloantibodies outside of RhD, which can cause hemolytic disease of the fetus and newborn (HDFN). For HDFN to occur to RhD, for example, during a first pregnancy, an RhD^- mother can become sensitized to RhD^+ RBCs from her fetus after exposure to fetal RBCs during fetomaternal hemorrhage either during labor or pregnancy. The ability of fetal RBCs to induce the development of maternal alloantibodies became evident after Levine and Stetson reported a case of a transfusion reaction in a woman who had recently bore a stillborn fetus and hypothesized that the woman had become immunized against an antigen from the dead fetus (16). Weiner and Peters then observed in 1940 that every recorded instance in which a hemolytic transfusion reaction followed a first transfusion occurred in pregnant or post-partum females (17). From these clinical observations, Landsteiner and Weiner immunized rabbits with blood from rhesus macaques. Agglutination assays of rabbit serum containing anti-rhesus (Rh) antibodies with human RBCs from sets of parents and children revealed the dominant inheritability of Rh and its presence on the RBC surface (18). While approximately 16% of Caucasians are RhD^- (19) and approximately 17% of RhD^- women become immunized after pregnancy with a RhD^+ fetus (20), the propensity of a RhD^- mother to develop anti-RhD alloantibodies is unpredictable. However, the consequences of these alloantibodies can be devastating. Anti-RhD IgG alloantibodies developed during the initial pregnancy (Fig. 1a) can cross the placenta during subsequent pregnancies causing fetal anemia, HDFN and potential fetal demise (Fig. 1b).

Despite knowledge of the RhD RBC antigen, the consequences of its genetic deletion (21, 22) and potential alloantibody development against it, prior to the 1960s, no

prophylaxis existed to actively prevent the development of these alloantibodies. In 1960, two groups of clinicians hypothesized that administration of anti-RhD alloantibodies from alloimmunized individuals may prevent the development of *de novo* anti-RhD alloantibodies. In England, this hypothesis was based on previous studies suggesting that fetal RBCs circulated in the maternal circulation (23-25). Moreover, in cases of ABO incompatibility between mother and fetus, anti-RhD alloimmunization was less likely (26, 27), suggesting that fetal RhD^+ RBCs may be removed by, for example, maternal anti-A alloantibodies recognizing A on the fetal RBCs prior to recognition of RhD by the maternal immune system (28). Furthermore, sensitization against RhD in RhD^- volunteers was also less likely when the RhD^+ RBCs were administered with ABO-incompatible RhD^+ RBCs or when ABO-compatible RBCs were coated with excess anti-RhD antibody prior to administration with RhD^+ RBCs (29). However, once sensitization against RhD occurs, no further protection is offered by ABO incompatibility between mother and fetus (30, 31).

The ability of maternal circulation to clear incompatible fetal RBCs was further supported by findings that anti-RhD-coated RBCs are sequestered in the spleen (32, 33) and that splenic macrophages were capable of transforming RBCs from flat disks into spherocytes by grasping the intact cell, leading to the idea that Fc receptors on macrophages may interact with antibody-coated RBCs (34). From these studies, Ronald Finn, J.C. Woodrow and C.A. Clarke (35-38) at the University of Liverpool sought to determine whether anti-A or anti-B antibodies may correlate with fetal RBC clearance from the maternal RBC circulation. Accordingly, Finn *et al.* demonstrated significant association between large fetal bleeds (>5 mL) in the maternal circulation and the

development of maternal anti-RhD. In cases where fetal RBCs were found in maternal circulation, mother and fetus were ABO compatible, whereas in cases where no fetal RBCs were found in the maternal circulation, mother and fetus were ABO incompatible, in support of previous studies. Furthermore, if the mother had increased number of fetal RBCs in circulation after delivery, she had an increased likelihood of developing anti-RhD alloantibodies (39), contributing to the ongoing thought that fetomaternal bleeding may be the most important factor in sensitizing RhD^- mothers (40).

Although clearance of fetal RBCs in the maternal circulation with radioactive labeling could not be tested, additional studies demonstrated that clearance of RhD^+ RBCs occurred when anti-RhD was administered to RhD^- males exposed to RhD^+ incompatible RBCs. To further identify the amount of anti-RhD-containing serum necessary to prevent alloimmunization in RhD^- males, men received 5 mL RBCs and 10 mL plasma. However, as eight of the thirteen men who received anti-RhD produced anti-RhD antibodies, while only one of eleven control recipients similarly produced anti-RhD, this ratio of RBCs to plasma actually resulted in enhanced alloimmunization. If more plasma (25-30 mL) was administered, then rates of alloimmunization decreased to three out of twenty-one anti-RhD treated compared to eleven out of twenty one controls that became alloimmunized (38). However, smaller doses of hyper-immunized volunteers could also be equally effective in preventing the development of anti-RhD alloantibodies in RhD^- males, suggesting that affinity maturation played a role in the ability of anti-RhD-containing plasma in preventing alloimmunization (41). From these studies, the University of Liverpool group concluded that fetomaternal hemorrhage primarily during delivery clearance exposed RhD^- mothers to RhD^+ RBCs. However, in the case of ABO

incompatibility, antibodies against major blood group antigens cleared the RhD^+ incompatible RBCs from the maternal circulation. Therefore, it seemed feasible that pooled plasma containing anti-RhD antibody could likewise clear RhD^+ fetal RBCs from the maternal circulation before an immune response could occur.

Another approach toward developing a prophylaxis against RhD alloimmunization was led by Vincent Freda, MD at Columbia-Presbyterian Medical Center in New York City. There, Freda, who incidentally was also the first American doctor to perform an amniocentesis in 1959 (42), hypothesized that anti-RhD antibody administered to an RhD^- mother at the time of delivery may prevent sensitization to avoid HDFN in her next child, with the ability of an antibody to inhibit the antibody response to an antigen termed antibody-mediated immunosuppression (AMIS). The rationale for AMIS as a potential hypothesis for the prevention of anti-RhD^+ sensitization was supported by studies demonstrating that production of diphtheria anti-toxin was decreased following combined immunization against diphtheria and pertussis in infants who had higher titers of passively transmitted antibodies at birth (43, 44).

Prior to testing this hypothesis in pregnant mothers, Freda and colleagues first administered anti-RhD IgG to prisoners at Sing-Sing Prison, eventually publishing his findings in 1964. During this trial, nine RhD^- males were transfused once a month with RhD^+ RBCs for five months in a row. Four of these nine prisoners also received anti-RhD IgG administration. Three months and six months after the last injection no circulating passive antibody could be detected in the prisoners administered anti-RhD IgG. In the control group, in which no anti-RhD IgG was administered, four out of five prisoners developed anti-RhD alloantibodies (45). In total, Freda demonstrated, together with Finn

and Woodrow, that passive administration of alloantibodies against RhD, later referred to as Rh immune globulin (RhIg), could prevent the development of an anti-RhD alloantibody response.

Following the discovery and 1967 publication of successful clinical trials that polyclonal anti-RhD administration could prevent the development of *de novo* anti-RhD alloantibodies to prevent HDFN (46), studies then sought to understand the potential mechanism governing the ability of RhIg to induce AMIS (Fig. 1c). Observations by Cooper *et al.* showed that infusion of hyper-immune agglutinating antibodies into rats caused spherocytosis and hemolysis, with a decrease in the hematocrit but no change in the mean cell volume, hemoglobin or red cell concentrations of potassium or adenosine triphosphate. Post-infusion, the membrane cholesterol, phospholipid protein and surface area decreased, but there was no change in the percentage composition of various RBC membrane phospholipids. This led the authors to conclude that perhaps spherocytosis-inducing agglutinating antibodies results in a loss of surface area in the RBCs with no accompanying change in hemoglobin or concentration of intracellular constituents, suggesting that perhaps a portion of the RBC membrane was removed (47). Applied to RhIg, these results further suggested that RhIg could selectively remove a portion of the RBC membrane.

Despite previous studies suggesting that antibody could induce alterations in the RBC membrane (47), supported by the close interactions observed between antibody-coated RBC and monocytes (34, 48), later studies of RhIg and AMIS instead focused on the ability of antibody to clear RBCs from the circulation. To study this, RBCs that were positive for both RhD and another RBC antigen, KEL, were chromium labeled and

transfused into KEL-negative males, along with administration of anti-KEL IgG antibody. Anti-KEL IgG induced rapid clearance of RBCs and prevented alloimmunization against RhD in thirty of thirty-one males treated with anti-KEL IgG. Additionally, given that anti-RhD could still agglutinate RBCs that had been treated *in vitro* with anti-KEL antibody, this led the authors to conclude that steric hindrance, whereby antibody against one antigen prevented binding of an antibody against another RBC antigen if the two antigens are in close proximity, was not a mechanism by which anti-KEL IgG prevented alloimmunization against RhD. Furthermore, these results suggested that AMIS occurred in a cell-specific, rather than antigen-specific manner as indicated by the clonal competition for antigen hypothesis (29), where the presence of a large number of antibody-forming cells for one RBC antigen may interfere with the antibody response to another. Although the clonal competition hypothesis was utilized to explain how anti-RhD alloantibodies were prevented when the RhD and ABO blood types both differed between donor and recipient, this hypothesis could also be applied to cases when there are multiple minor antigens on the RBC surface.

In order to define the specific components within RhIg that may be important for AMIS, studies began to focus on the ability of particular subsets of antibody within the polyclonal mixture of RhIg to induce AMIS, rather than the serum as a whole. Early studies showed that IgG3 antibodies were more efficient than IgG1 antibodies in the destruction of RBCs (49), that the severity of HDFN was greater in the presence of IgG3 anti-RhD and high monocyte-mediated RBC lysis in the maternal serum (50) and that the severity of HDFN may be correlated with IgG1 rather than IgG3 (51). Other studies indicated that severe HDFN was characterized primarily by the development of maternal

IgG1 and IgG3 (52). Regardless, these studies therefore suggested that if clearance of fetal RhD^+ RBCs before development of a maternal anti-RhD alloantibody response was the primary mechanism behind AMIS (45, 53-57), anti-RhD IgG1 and IgG3 might be more effective in suppressing anti-RhD alloimmunization.

Given that RhIg was produced either from sensitized individuals immunized through pregnancy, incidental transfusion or purposeful administration of RhD^+ RBCs into RhD^- recipients, the availability of RhIg was dependent on available sensitized donors. Additionally, although purposeful administration of antigen-positive RBCs into antigen-negative recipients was possible for the RhD antigen, this is not a feasible nor ethical approach for other RBC antigens, as alloimmunization against many RBC antigens can cause difficulty in finding compatible RBC units should the individual need RBC transfusions in the future and could place these volunteers at risk for hemolytic transfusion reactions. Therefore, to circumvent these potential problems and better study how IgG1 and IgG3 within RhIg may be effective in suppressing AMIS, anti-RhD monoclonal antibodies were produced (58-60) by fusing B-lymphoblastoid cells with anti-RhD-producing B cells from alloimmunized donors to produce a monoclonal anti-RhD secreting cell line, leading to several *in vitro* and *in vivo* studies to try to elucidate the molecular mechanisms governing AMIS.

Utilizing these monoclonal anti-RhD monoclonal antibodies, IgG1 or IgG3 anti-RhD secreting clones were tested for ability to bind to O^+ RBCs and induce rosette formation on a human monocyte cell line, whereby antibody-saturated RBCs bind lymphocytes presumably through interactions with Fc receptors on the lymphocyte in a petal-like formation. Anti-RhD IgG3 was able to induce rosette formation via interaction

with Fcγ receptor I (FcγRI); decreasing FcγRI expression caused decrease rosette formation but increasing FcγRI expression (with IFNγ) increased rosette formation. From this study, the factors proposed to be important for interaction between antibody-coated target cell and effector cell included: density of antibody on target cells, density of FcγRI on effector cells and availability of interaction sites on IgG (61). Given IgG3's ability to induce rosette formation, the authors hypothesized that the extended hinge region of IgG3 was able to overcome low antibody and FcγRI densities, as well as charge repulsion on surface of interacting cells—a comparison between anti-RhD IgG1 and IgG3 that would continue through a variety of biochemical studies utilizing these monoclonal antibodies (62).

Further comparisons of the *in vitro* activities of monoclonal anti-RhD IgG1 and IgG3 utilized phagocytosis by monocytes as a surrogate for clearance *in vitro* and extrapolated the results of *in vitro* studies with the anti-RhD monoclonal antibodies to suggest what their activity may be *in vivo* (58). In an assay that measured erythrophagocytosis resulting in the production of reactive oxygen species that react with luminol to produce light, a lower concentration of IgG3 antibody was able to sensitize RBCs for monocytes, resulting in enhanced chemiluminscence in RBCs sensitized with IgG3 compared to IgG1 (63). Whereas anti-RhD IgG3 monoclonal antibody mediated greater adherence to RBCs and lysis of RBCs by monocytes compared to anti-RhD IgG1 (58, 62, 64), IgG1-sensitized RBCs underwent greater phagocytosis *in vitro* when incubated with monocytes compared to IgG3-sensitized RBCs (65). Given the different findings in the ability of anti-RhD IgG1 and IgG3 to mediate RBC adherence, lysis and phagocytosis *in vitro*, these results indicated that induction of AMIS by RhIg may require

the IgG1 for phagocytosis but IgG3 for rapid clearance (66, 67). These results suggest that perhaps future preparations of RhIg could include blends of anti-RhD IgG3 and IgG1 to induce the desired RBC clearance to induce AMIS (68).

To correlate these *in vitro* findings with potential implications *in vivo*, serum samples from 131 pregnant alloimmunized women, 75 of whom developed anti-RhD alloantibody, were assessed for how *in vitro* assays correlated with the severity of HDFN in the patients. Hadley *et al.* (69) found that monocyte-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and the production of chemiluminescence as a marker for monocyte activation best correlated with the severity of HDFN; however, these results did not indicate whether chemiluminescence nor monocyte-mediated ADCC could predict the later development of HDFN. Although other studies would attempt to elucidate the role of glycosylation and enzyme treatment of anti-RhD monoclonal antibodies to determine whether aspects of antibody could be altered to learn more about the biochemical properties of these monoclonal antibodies and their ability to induce AMIS (70-74), these studies did not seem to offer conclusive indications that a single property was important for the ability of a particular monoclonal antibody to induce AMIS.

Because previous *in vivo* studies of RhIg indicated that clearance of RhD^+ RBCs before a recipient immune response occurs may be the primary mechanism behind AMIS and *in vitro* studies could not directly measure clearance, Kumpel *et al.* assessed whether IgG1 and IgG3 monoclonal anti-RhD antibodies, referred to as BRAD 5 and BRAD 3, respectively, could induce AMIS *in vivo* and whether this correlated with RBC clearance (75). To study this, the ability of polyclonal RhIg and monoclonal anti-RhD antibodies

(BRAD 3 and BRAD 5) were assessed for their ability to clear chromium-labeled RhD^+ RBCs following an initial transfusion, followed by challenges with unlabeled RhD^+ RBCs at 6 and 9 months post-administration of monoclonal anti-RhD with chromium-labeled RhD^+ RBCs. Although there was no development of anti-RhD alloantibody at 6 months, five out of twenty-four subjects eventually produced anti-RhD antibodies after one or two further exposures to RhD^+ RBCs. Furthermore, although a majority of anti-RhD monoclonal antibody-administered individuals did not produce any detectable anti-RhD alloantibodies, there was substantial variability in the rates of RBC clearance. For example, at 24 hours, individuals who received BRAD 3 had between 1.1% to 58% circulating RhD^+ RBCs, which did not provide conclusive evidence that the RBC clearance rates related with the later development of anti-RhD alloantibodies (75). This variability in clearance rates continued in other studies, when, for example, administration of rat monoclonal antibody derived from Chinese hamster ovary cells resulted in extremely variable red cell clearance with no correlation to the dose of anti-RhD antibody administered (76), although administration of this antibody did result in AMIS. In another study, monoclonal antibodies from murine myeloma cell lines resulted in not only variable clearance, but also enhanced anti-RhD alloimmunization rates (20, 77-79). From these studies, it became evident that RBC clearance was not likely the primary mechanism whereby AMIS occurs, as the rates of RBC clearance did not consistently correlate with whether a particular antibody could induce AMIS.

To further elucidate the molecular mechanisms governing AMIS, several murine models of RBC alloimmunization were developed to take advantage of the ability to delete single genes to isolate particular immunologic pathways that may be important in

AMIS. However, these mouse models have shown different results, depending on the RBC antigen used. In one model system AMIS appears to occur through antibody-induced antigen modulation that requires complement fixation and Fcγ receptor engagement (80). In contrast, in another model system, Fcγ receptors but not complement fixation were required for antigen modulation to occur (81, 82). Furthermore, while these two antigen systems implicate the role of antigen modulation in AMIS albeit through different mechanisms, in a third model system, AMIS via antigen modulation occurs in the absence of inhibitory or activating Fcγ receptors (83, 84), suggesting that while antigen modulation may occur through a variety of different pathways, antigen modulation itself may serve as a surrogate to screen the therapeutic capacity of antibodies for potential ability to induce AMIS. Given that each model system undergoes antigen modulation and RBC clearance through different mechanisms, the mechanism of AMIS for each RBC antigen may differ. As result, it is possible that one mechanism may not govern AMIS against all RBC alloantigens.

CONCLUDING REMARKS: AMIS

Over time, RBCs evolved from merely a humor that could potentially bring strength to those who drank them to a therapeutic intervention during a time of crisis. However, transfusion of RBCs was not without risk, as RBCs possess not only ABO antigens but also other alloantigens, such as RhD. In the setting of pregnancy, RhD can potentially cause the development of HDFN in fetuses born to alloimmunized mothers. However, with the advent of RhIg, the ability to prevent the development of anti-RhD alloantibodies using pooled donor serum was realized. However, while studies with both

polyclonal and monoclonal anti-RhD antibody mixtures have suggested that antigen modulation may be one potential mechanism behind AMIS, more studies beyond this dissertation will be necessary to better elucidate how antibodies against an antigen can suppress the development of *de novo* antibodies to that target antigen. Furthermore, as RhD does not fix serum complement components (85), yet complement binding has been implicated not only in the development of a humoral response (86-89) and antibody-mediated hemolytic transfusion reactions (85), but also in regulating AMIS to the one model RBC antigen (80), further insight into the role of complement in RBC alloimmunization to alloantigens other than RhD is necessary.

INTRODUCTION TO COMPLEMENT AND HISTORICAL PERSPECTIVE

As will become evident from the following description of complement's discovery, complement and RBC biology are intimately related, as RBCs were primarily utilized to elucidate the components of the complement pathway. Indeed, over 120 years ago, early experiments on what would become known as "complement" by George Nuttall first observed how sheep RBCs (SRBCs) mixed with anthrax bacilli had bactericidal activity that was lost when the SRBCs were heated. This observation was counter to Elie Metchnikoff's earlier supposition that the bactericidal properties of immunity depended on phagocytes. Work by Hans Buchner in 1889 not only confirmed the observations made by Nuttall, but also renamed the bactericidal component in blood serum "alexin," derived from the Greek word *alexos*, meaning to "ward off" (90). Later in 1894, Richard Pfieffer observed that blood from guinea pigs, which had recovered from cholera infection, could be mixed with bacteria and injected into the peritoneal

cavity of normal guinea pigs, conferring protection from a later infection with cholera. Pfeiffer also saw that lysis of the bacteria could be seen by removing successive dilutions of peritoneal fluid from the guinea pigs, post-immunization, termed the "Pfeiffer phenomenon"(90). Building upon the earlier work by Nuttall and Buchner, Jules Bordet, who would later win the Nobel Prize in Physiology and Medicine in 1919 for his work on complement, among other aspects of immunity, is now credited with performing the critical experiments associated with the discovery of complement (91), where he identified the thermosensitive component of complement (92). Thus, the late 19th century through the early 20th century was marked not only by observations about complement's existence, but also its role in protective immunity.

From the initial experiments that confirmed complement's bactericidal properties, later observations would show that complement contained multiple components—some of which were heat-stable and others that were heat-labile. It was not until 1899 that Paul Ehrlich, who would later win the Nobel Prize in Physiology and Medicine in 1908, renamed the heat-labile alexin as "complement" and the complementary heat-stable portion that interacts with complement as "amboceptor," which we now know as antibodies. The amboceptor, he hypothesized, had two binding sites, one for the bacteria and the other end that binds to complement. Although Ehrlich proposed incorrectly that the blood contained many different types of complements with a variety of functions, rather than a unified cascade, his ideas about amboceptors' interaction with complement represented an early rendition of the classical pathway of complement activation. Ehrlich's initial observations about the classical pathway were confirmed in 1907, where Adolfo Ferrata dialyzed serum in an isotonic solution and saw that the serum split into

two components, named *Mittlestuck* (mid-piece) and *Endstuck* (end-piece) in German by Brand, who also repeated Ehrlich's experiments. In an isotonic salt solution, neither component alone caused hemolysis, but it was only when the components were mixed together that complement activity was restored (93). Several subsequent scientists sought to biochemically describe the splitting of complement into end-piece and mid-piece, such as the observations that complement loses its activity in distilled water, along with isotonic sugar, or that splitting of complement could be caused in a solution of hydrochloric acid (93). Suffice it to say that complement splitting was of much biochemical interest to scientists in the early 20th century. Consequently, over the course of the early part of the 20th century, the focus in complement biology shifted from identifying complement itself to trying to understand how the parts related to each other in terms of its split products.

Before the different components of the complement cascade were discovered, it was already apparent that complement was a part of innate immunity. Indeed, it was well-known even in a 1918 *New York Medical Journal* article that complement is non-specific and a component of blood serum, separate from RBCs and lymphocytes (90). Before the components of complement were identified, the temperature-sensitive nature of complement was determined by storing guinea pig serum at temperatures ranging from 4°C to 37°C and observing its ability to hemolyze SRBCs (92). Additionally, Morrison directly challenged Metchnikoff's hypothesis that alexin was leukocytic in nature by pipetting off the buffy coat of clotted blood and observing that complement-mediated lysis still occurred. By 1927, the complement system was defined as containing three components: a heat labile component from the antibodies of serum (mid-piece), a heat

stable component that associates with the globulins (also comprises the mid-piece), and heat labile components containing proteins (described as albumins) of the serum (end-piece) (94). Thus, complement became known as not a single entity, but a conglomerate of proteins with unique functions.

Although it was not well understood how the different parts of the complement system related to each other in a proteolytic cascade, it became apparent that multiple components were produced through biochemical methods of splitting. For example, Whitehead, Gordon and Wormall observed that a heat-stable fourth component of complement existed through splitting of guinea pig complement via ammonia (94). Additional delineation of the components of complement occurred in 1941, when L. Pillemer and E.E. Ecker examined the electrophoretic migration patterns of normal guinea pig serum. Based on the migration pattern of the serum proteins and which reagents could destroy each component, they purported that the terminology of complement should be changed from descriptive terms of the thermostability of various components to a numerical designation based on the order in which each component was discovered: C1, C2, C3 and C4, referring to complement components 1-4, respectively (95), which we now continue to use today.

Progression in terms of complement's terminology to better explain the parts of the cascade and studies on characterizing various components of the complement cascade also allowed for depletion of specific components of complement from the serum to better understand the role of each protein. More specifically, treatment of serum with ammonia depleted C4, while treatment of serum with zymin or a carbohydrate from fresh yeast could deplete C3. C1 and C2 could be depleted from serum simply by heating the

serum to 56°C (96). Further studies on C4 using SRBCs fixed with guinea pig complement via anti-pneumococcus antibody found that inactivation of C1 and C2 in guinea pig serum resulted in inhibited complement fixation of C3 and C4 (96). Conversely, inhibition of C3 and C4 did not affect fixation of C1 and C2, indicating that C1 and C2 act prior to C3 and C4. By studying the different amounts of C1 able to fix RBC aggregates, Pillemer and Ecker also concluded that guinea pig was superior in its C1 fixation than sheep, human and dog complement, creating a scientific trend in which guinea pig complement would primarily be used in further studies of complement. Furthermore, in this study, Pillemer and Ecker concluded that C2 forms a complex with C4 and that this C4-C2 complex, together with C1, must first complex with antibody and antigen, before the cascade converges on C3 (95). Thus, by the early 1940s, it was evident that the complement system may not simply be an aggregation of proteins, but rather a more complex cascade, in which certain proteins act in a defined sequence.

Although Pillemer and Ecker proposed that the complement system is a cascade, it was not until Mayer *et al.* developed a spectrophotometric method in 1948 of titrating rabbit complement with SRBCs that they came to better understand the interplay between temperature, time, antibody concentration and complement concentration in the complement cascade. Their method also relied on the ability to inhibit complement fixation at any time with citrate, which contained magnesium and calcium (97). Using this method, Mayer, along with Lawrence Levine and Herbert Rapp, published a series of several articles in *The Journal of Immunology* (97-102), detailing each step in the complement system. Through these articles, they described what was previously known as C_x and C_y into C2 and C3, which act sequentially. They also found that while C2

derived from rabbit serum requires magnesium, C3 does not (102). The complexity of the complement system was further described by Amiraian *et al.*, who hypothesized in a 1958 *Science* article that lysis of cells that have interacted with complement proteins seems to rely on another component other than C3, whose properties do not coincide with C1, C2 or C4 (103). Separation of the components of guinea pig complement by an N, N-diethylaminoethyl cellulose column in 1959 confirmed the presence of these same components (C2, C3, C_a and C_b) in guinea pig serum and their stability post-fractionation, but did not address the idea that further factors existed. Furthermore, Rapp also showed that C3 is actually composed of two proteins, which we now know as C3a and C3b (104). Consequently, from the studies by Mayer, Rapp and colleagues, it became apparent from a biochemical perspective that the complement system consisted of more components than originally defined.

Building upon Mayer, Levine and Rapp's kinetic analysis of complement fixation, Weinrach *et al.* expounded upon the need for antibody in complement fixation. To accomplish this, Weinrach and colleagues used chromium labeling to measure the sensitization of SRBC to complement fixation via different concentrations of antibody and serum. Using a series of titration experiments and early mathematical modeling they concluded that complement fixation by antibody is characterized by a rapid hemolysis phase, followed by a slower logarithmic phase perhaps due to random movement of antibody over the red cell surface until chance encounter with complement components (105, 106). Consequently, they hypothesized that these kinetic studies could be used to determine the optimum hemolytic efficiency of an antibody based on turnover time and avidity.

Further descriptions of the antibody-complement complex would later be elucidated in 1965 when Borsos *et al.* published dose response curves comparing the ability of the 19S fraction of antibody (IgM) to fix complement to the 7S fraction of antibody (IgG) by incubating the two fractions with SRBCs and guinea pig complement, referred to as the "C1a fixation and transfer test." Different combinations of antigens and IgM or IgG antibodies were incubated to determine the antibody concentration and C1a concentration necessary to cause complement fixation, including: (i) SRBCs and rabbit antibody to SRBCs, (ii) *Salmonella typhosa* and rabbit antibody to *S.* typhosa, (iii) *S. typhosa* "O" antigen and rabbit antibody to "O" antigen, (iv) *E. coli* and rabbit antibody to *E. coli*, (v) human RBCs with blood type O and human serum containing "cold agglutinin," and finally (vi) human RBCs with blood type A and human antibody to blood group A. Ultimately, Borsos and Rapp concluded that while a single molecule of 19S antibody (IgM) is capable of fixing C1a, two molecules of 7S antibodies (IgG) in close proximity are required to fix C1a (107), thereby elucidating a contemporary model of the classical complement pathway. Characterization of the components of complement in human serum began when scientists could relate proteins in human serum to the known complement proteins in guinea pig and rabbit serum. More specifically, in 1959, Muller-Eberhard *et al*. described similarities between one of four β-globulins in human serum and the R_3 (also known as C3) component of guinea pig serum. After isolation of β_{1C}-globulin from human serum via anion exchange chromatography, β_{1C}-globulin was found to become inactivated at 63°C, similar to other complement components, but unlike the other more heat stable β-globulins. Furthermore, they found that incubation of β_{1C}-globulin with R_3 from guinea pig serum rescued its hemolytic activity, and incubation of

β_{1C}-globulin with R_3 from human serum increased its hemolytic activity of SRBCs, leading the authors to conclude that β_{1C}-globulin is perhaps the human homologue of guinea pig C3 (108). Aside from the early identification of C3 as β_{1C}-globulin in human serum, later descriptions of complement components in human serum occurred through biochemical assays in the 1960s and 1970s. For example, C1 was demonstrated to be a complex of individual parts (C1q, C1s, C1r respectively) in normal human serum through single radial immunodiffusion analysis, in which serum could be run via agar gel electrophoresis that had been incorporated with antibody. As the antigen (C1 in this case) ran through the gel, precipitin complexes of antibody and antigen formed. In Ziccardi *et al*., the formation of multiple precipitin rings when normal human serum was incubated with EDTA and indicated that EDTA was able to cause dissociation of C1 into its subunit complexes. However, in the presence of calcium, C1 in normal human serum remained intact. Through single radial electrophoresis, Ziccardi *et al*. also showed that free C1s could be detected in the serum, providing a potential therapeutic application if patients perhaps had free C1s in their sera (109). Thus, by the end of the 1950s, elucidation of the components of complement in human serum not only allowed for a cross-species comparison of the complement components, but also enabled further research into the role of complement in human immune responses.

In addition to isolation and characterization of C1, C3 was also isolated and characterized from human serum in 1969, Bokisch *et al.* cleaved C3 into C3a and C3b then C3b into C3c and C3d, by trypsin. They also described the ability of C3a to promoting polymorphonuclear cells chemotaxis and increase vascular permeability in guinea pig and rabbit skin. Finally, Bokisch *et al.* also showed that the chemotaxis and

contraction induced by C3a could be inhibited by anti-C3a (110). Hugli *et al.* described the peptide sequence of C3a in 1975, when they showed that human C3a is highly cationic and identified its sequence by overlapping peptides following cleavage by cyanogen bromide, as well as trypsin and chymotrypsin. In particular, cysteine residues were identified by alkylation with bromoacetate and C3a was found to be resistant to extreme pH and heat. Edman degradation was used to identify C3a's peptide sequence from overlapping peptide portions. The structure of murine complement component C3, specifically the alpha chain, would later be sequenced and cloned from a murine liver cDNA library (111). While these studies described C3a's peptide sequence and biological properties, further studies were necessary to confirm how structure impacted C3's function as a linker between different parts of the complement pathway.

In addition to earlier studies by Bokisch *et al.* and Hammer *et al.* characterizing not only C3a's structure but also its chemotactic properties, C3 and its fragments became characterized as a critical link between different complement pathways to converge upon formation of the membrane attack complex. Further describing the order of the complement cascade, Lachman *et al.* demonstrated that what was previously called the "activated reactor" was actually a complex formed by C5-C6, which then formed a complex with C7. C7 could then attach to RBC membranes, leading to activation of C8 and C9, demonstrated by incubating dilutions of C7 with anti-C3, C4, C5, C6, C7, and C8, IgG, serum and RBCs, causing lysis. Consequently, the attachment of C7 and recruitment of C8 and C9 thereby formed what is now known as the membrane attack complex, leading to the phenomenon referred to as "reactive lysis" (112). Hammer *et al.* described C3 as a central component of the complement pathway when the hemolytic

activity of C5-C6 was shown to be enhanced on SRBCs only when bound by guinea pig C3b and rabbit antibody, compared to RBCs alone. Titration studies of C5-C6, along with titrations of C3b separately found that the hemolytic activity of complement was dependent on both factors together (113). Therefore, by the mid-1970s, the schematic of complement began to unfold, but the biological and immunological consequences of the cascade remained yet to be defined.

More insight into the evolutionary parallels between C3 in humans and C3 in other animals, as well as the ability to utilize these parallels in experimental settings, became evident in studies with cobra venom factor. Although it had been hypothesized since 1902 that snake venom had bacteriolytic and hemolytic properties, in which Simon Flexner, MD and Hideyo Noguchi, MD wrote, "There is conclusive experimental evidence that, although the intermediary body unites first with the cells—bacterial, blood cells, etc.—this substance by itself cannot bring about solution. But after the union of this intermediary body [i.e. antibody] with the cells the complement is capable of being brought into action, through this intermediation, so that solution takes place." Flexner and Noguchi also demonstrated that heating serum to 56-58°C destroyed complement but not the intermediary body, thus inhibiting the venom's bacteriolytic and hemolytic capabilities (114). However, the exact biochemical nature of snake venom and its relation to complement was not shown until 1976 when Alper *et al*. demonstrated that when cobra venom reacted with normal human serum, cobra venom migrated more quickly on an agarose gel by gel electrophoresis, compared to cobra venom that reacted with C3-deficient serum, indicating that cobra venom was altered after reacting with a yet unknown molecular factor in the serum (115). Immunofixation revealed that cobra venom

factor showed a series of bands after reacting with normal human serum, as compared to one sharp band prior to reacting, again suggesting that the cobra venom factor was altered post-reaction, perhaps by proteolytic enzymes. Additionally, incubation of cobra venom factor with whole cobra serum reduced the ability of cobra venom factor to alter C3b in human serum, perhaps suggesting a finite ability of cobra venom factor to react with human serum (115). From these studies, cobra venom factor was found to be analogous to human C3b, thus shedding light on a tool that would be used in multiple studies of C3, (115-121) even to this day.

From the aforementioned studies starting in the late 1800s to the 1970s, a simple observation regarding bacterial lysis associated with animal serum morphed into one of the fundamental concepts of the innate immune system: the complement cascade. Although the elaborate nature of the complement system with its proteolytic and multi-component complexity was beginning to be appreciated from biochemical assays, complement's involvement in both humoral and cellular immunity would be elucidated in the coming years. As such, the following sections will describe how complement biology evolved into a dynamic field, responsible for not only linking the innate and adaptive immune system, but also functionally diverse in its involvement in both B and T cell responses.

COMPLEMENT AND HUMORAL IMMUNITY

Throughout the 1980s and 1990s, complement biology would become dominated by two important players, one of whom was Douglas Fearon of Cold Spring Harbor Laboratory and a member of the National Academy of Sciences, from whose lab came

the description of complement's role in humoral immunity as a "molecular adjuvant." The other powerhouse complement lab belonged to Michael Carroll at Harvard University, from whose lab comes much of our understanding of the immune response in complement-deficient and complement receptor-deficient animals. Prior to the rise of Fearon and Carroll, Pepys *et al.* described that while it was known that B cells have a membrane receptor that engages C3, it was not known whether there is a direct link between C3 and the antibody response to an infection. To study whether C3 may impact antibody development in the context of an immune challenge, Pepys *et al*. depleted C3 in Balb/c mice using cobra venom factor and then examined the immune response to immunization with SRBCs and pneumococcal polysaccharide type III. If C3 was depleted with cobra venom factor prior to challenge with SRBCs, the antibody response to SRBCs was decreased. However, if SRBCs were injected prior to C3 depletion with cobra venom factor, 3/11 mice had decreased antibody responses, but the other mice had a relatively normal immune response compared to non-depleted mice, indicating that not only was C3 critical to the development of an antibody response to SRBCs, but also that the timing of C3's involvement was important as well. On the other hand, when mice were exposed to streptococcus polysaccharide III after depleting C3 with cobra venom factor, there was no impact on the antibody response to streptococcus polysaccharide, suggesting that C3 did not play a role in the antibody response to streptococcus polysaccharide, which we now know is a T-independent antigen. Finally, in an allograft skin graft model, complement-depleted mice had grafts with increased survival (122). Thus, these findings implicated complement in the development of humoral immunity, which would become the major focus of complement biology for the next 25 years.

Providing more biochemical insight into protein-receptor interactions in the complement pathway, in 1981, Lambris *et al.* identified C3d binding protein as complement receptor 2 (CR2) when SRBCs were found to bind CR2 when C3d was bound to the surface but not C3b. Although it was previously known that CR2 expression is limited to B cells, CR1 is also expressed on monocytes, neutrophils and T cells in humans. Based on these previous findings, Lambris *et al.* immunized rabbits with C3d binding protein and found that it inhibited lymphocyte rosette formation with SRBCs bound to C3d, but not C3b. From the knowledge garnered by C3d binding protein's molecular weight and its ability to inhibit rosette formation, Lambris *et al.* concluded that C3d binding protein is actually membrane-shed CR2 from B cells based on C3d binding protein's molecular weight (123). However, it was still unclear how C3d binding protein, CR2, may contribute to B cell signaling and activation. Therefore, based on the biochemical pathway of B cells, in which IgM cross-linking induces phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), leading to activation of protein kinase C, Carter *et al.* from the Fearon Lab sought to understand how CR1 and CR2 interactions on B cells may potentially modulate calcium flux. He induced calcium flux in tonsillar cells by cross-linking by anti-IgM, anti-CR2 or anti-CR1 with goat anti-mouse IgG and observed enhanced calcium flux. More specifically, he observed increased ^{3}H thymidine incorporation with CR2 and IgM, thus providing a direct biochemical link between CR2 and B cell activation (124) and a basis for what would later be referred to as the Carter and Fearon threshold model for how binding of C3 to antigen reduces the level of antigen required for B cell activation (125). Further linking components of the complement pathway and B cell activation was Thornton *et al.* who

demonstrated that keyhole limpet hemocyanin (KLH), a model antigen, leads to immune complex formation with C3 that then can bind to CR2 on B cells. KLH immune complex interaction with CR2 consequently caused upregulation of CD90 on peripheral blood mononuclear cells. In addition to showing co-stimulatory molecular upregulation upon B cell interaction with KLH-complement-immune complexes, Carter *et al.* also demonstrated that although KLH immune complexes can be phagocytosed and presented on major histocompatibility complex II by antigen-specific and non-specific B cells, only KLH-specific T cells were then activated (126). Therefore, based upon the work of Lambris, Carter and Thornton, by the mid-1990s, the biochemical linkage between complement components and B cell activation was established.

Although the protein-protein interaction between spliced components of C3 and CR2 was known, a more defined mechanism behind why complement was important for the development of an antibody response was less characterized. In 1996, Dempsey *et al.* observed that HEL fused to two to three copies of C3d was more immunogenic than HEL alone (127). To determine the concentration of HEL-C3d necessary to elicit an immune response, Dempsey and colleagues performed competitive binding assays, where iodine-labeled HEL-C3d competed for binding to CR2 with HEL-C3d alone. Through their competitive binding assays and by comparing the concentration of HEL emulsified in Complete Freud's Adjuvant (CFA) versus HEL-C3d diluted in PBS capable of eliciting an IgG1 response, they described a threshold effect where once a certain concentration of either HEL-CFA or HEL-C3d was reached, an IgG1 response could be elicited. Furthermore, Dempsey *et al.* also described how the adjuvant effect of C3d was specific to what was bound to the antigen, in that when mice were immunized with KLH then

challenged with HEL-C3d, only an antibody response against HEL-C3d was observed. Together, these studies demonstrated that although complement was previously described as a primitive system, it could not only differentiate between harmful and innocuous stimuli, but could also act as a "molecular adjuvant" to positively regulate B cells in the development of humoral immunity.

A direct application of Dempsey's model of C3d as a "molecular adjuvant" would emerge when Ross *et al.* published a *Nature Immunology* paper using a DNA vaccine encoding a fusion of C3d with hemagglutinin (HA) from *Haemophilus influenzae.* Ross and colleagues found a similar IgG response to influenza in mice immunized with a DNA vaccine expressing transmembrane HA or C3d-HA plasmid with higher avidity antibody observed at weeks 8 and 14 post-infection. However, the mice immunized with the C3d-HA vaccine did experience a protective effect from weight loss after lethal influenza infection. One hypothesis of the mechanism behind the C3d-HA DNA vaccine was that the fused C3d component of the vaccine facilitates binding of the HA antigen to follicular dendritic cells in the germinal center, where affinity maturation occurs (128). Consequently, Dempsey *et al.*'s idea of C3d as a "molecular adjuvant" with the ability to modulate a threshold for B cell activation revolutionized complement biology and had consequences not only for antibody responses against model antigens, but also in vaccine design.

Supporting Carter and Fearon's model that binding of C3 to antigen reduces the threshold of antigen required for B cell activation was Fischer *et al.* from the Carroll Lab, who sought to understand how humoral immunity was altered in the setting of mice deficient in C3 (C3 KO). To determine whether the humoral and adaptive immune

response changed in C3 KO mice, Fischer *et al.* immunized wild-type and C3 KO mice with a T-dependent antigen, bacteriophage ΦX174, then examined the antibody response between wild-type and C3 KO mice in terms of germinal center formation, isotype switching and B cell signaling. 10 days after immunization, C3 KO mice had smaller germinal centers, as seen through immunofluorescence with peanut agglutinin (PNA), a marker for germinal centers. They also found that C3 KO mice failed to isotype switch, but had normal B cell proliferation, as measured through ^{3}H thymidine incorporation, after *ex vivo* stimulation with anti-IgM, soluble CD40L, or soluble CD40L and recombinant IL-4 together. Adoptive transfer of T cells from C3 KO mice into wild-type mice revealed normal antibody responses, showing that while T cell function is intact in C3 KO mice, B cell function is not (129). Thus, Fischer *et al.* demonstrated that C3 was important to the development of a robust immune response, a finding that would be referenced repeatedly and replicated using other antigens.

In a separate study examining the response in C3 KO mice to bacteriophage, Fischer *et al.* then investigated whether the antibody response in C3 KO or mice deficient in C4 (C4 KO mice) depended on the antigen itself. When C3 KO mice were exposed to endotoxin derived from gram-negative bacteria, often implicated as a cause of sepsis in patients, they experienced increase mortality, which could be corrected with an injection of C3 serum from humans. Furthermore, C4 KO and C3 KO mice injected with endotoxin also had higher levels of endotoxin, indicating a role of the complement cascade in clearing endotoxin. Although one to two hours post-infection, C3 KO and C4 KO mice had a peak in their serum tumor necrosis factor (TNF) and IL-1β, perhaps indicating a “cytokine storm” associated with their septic state, C3 KO and C4 KO mice

had no difference in nitrous oxide production, indicating no difference in a breakdown in reactive oxygen species. Providing more mechanistic insight into why C3 KO and C4 KO mice experienced increased mortality due to infection with endotoxin, Fischer *et al.* observed increased C1 inhibitor (C1INH) consumption in C3 KO and C4 KO mice during infection. Because C1INH is an important regulator of factors XII, XI and kallikrein of the clotting cascade, Fischer hypothesized that consumption of C1INH by C3 KO and C4 KO mice was contributing to their dysregulation of factors in the clotting cascade, leading to disseminated intravascular coagulation (DIC) typically seen in the setting of sepsis. C1INH supplementation with purified mouse C1INH improved survival in the infected C3 KO and C4 KO mice (130). Consequently, with Fischer's studies in C3 KO mice in 1996-1997, complement biology shifted from focusing on the biochemical and molecular interactions of complement proteins to how the immune response was altered in the setting without complement protein or complement receptor expression.

Although a majority of complement components are synthesized in the liver and a majority of the aforementioned papers concentrate on liver-derived complement components, some proportion of complement is synthesized in other peripheral organs. Other sources of complement include keratinocytes, kidney tubular epithelial cells and macrophages. To determine if myeloid C3 is important in the development of a humoral immune response, Fischer *et al.* from the Carroll Lab reconstituted C3 KO mice with wild-type bone marrow to restore C3 derived from hematopoietic cells, which corrected humoral defects seen when C3 KO and wild-type mice were immunized with KLH. Fischer and colleagues also found that the major source of C3 mRNA was $MOMA2^{+}$ macrophages in the white pulp of the spleen and that local synthesis by donor

macrophages reversed impaired antigen trapping by splenic follicular dendritic cells (FDCs) (131), thus representing one of the first demonstrations that hematopoietic-derived cell synthesis of C3 may play a role in the humoral response to a given antigen.

Further elucidating the role of peripheral sources of C3, other than the liver, Verschoor *et al.,* also from the Carroll Lab, developed a radiation chimera, where all sources of C3 remained intact, except for bone marrow-derived cells, which were C3 deficient. Chimeric mice were created by reconstituting lethally irradiated wild-type or C3 KO mice with wild-type bone marrow. A reverse chimera was also created, where lethally irradiated wild-type mice were reconstituted with C3 KO bone marrow, but had normal C3 in non-hematopoietic derived cells. When the chimeric and reverse chimeric mice were infected with herpes simplex virus (HSV), C3 KO mice reconstituted with WT bone marrow exhibited normal anti-HSV antibody development, while WT mice reconstituted with C3 KO bone marrow had a decreased antibody response to HSV infection and decreased FDC migration to lymph nodes. Although the antibody response in mice reconstituted with C3 KO bone marrow was decreased, the $CD4^+$ T cell response to HSV, and leukocyte and $CD11b^+$ (referring to monocytes and macrophages) infiltration into peripheral lymph nodes remained intact. Interestingly, the antibody response seemed dependent on the route of administration, in that intravenous administration of HSV resulted in a normal antibody response in the reconstituted with C3 KO bone marrow, while intradermal administration of HSV resulted in a decreased antibody response in the reverse chimeric mice (132). Thus, from these studies, we now know that complement is not only synthesized by hepatocytes, but also by cells of

hematopoietic-lineage, which then have an impact on the development of an anti-viral response.

Also emerging from the Carroll Lab in 1996 was another high impact study that would further shift the techniques in complement biology more towards the utilization of knockout models, when Ahearn *et al.* sought to understand the mechanism of how covalent attachment of C3 components to antigen enhances immunity. Instead of knocking out C3, which attaches to antigen and allows binding to CR2, Ahearn and colleagues targeted CR2 instead. In mice, CR2 is encoded by one gene for CR1 and 2 (CR1/2) and then alternatively spliced to form CR1 or CR2. Following targeted deletion of the CR2 locus in mice, Ahearn *et al.* infected mice deficient in CR2 (CR1/2 KO) with T-dependent antigen, bacteriophage ΦX174, and examined antibody development, germinal center formation and B cell signaling, similar to Fischer *et al.*'s earlier work. Akin to the disruption of antibody responses in C3 KO mice infected with bacteriophage ΦX174, CR1/2 KO mice also experienced decreased antibody responses and decreased germinal center formation, as shown through immunofluorescence. Furthermore, Ahearn *et al.* also observed a decrease in the number of peritoneal B1a cells ($CD5^+$ IgM^+) when CR2 was disrupted. Analogous to C3 KO mice infected with bacteriophage ΦX174, CR1/2 KO mice showed no defect in signal transduction via their B cell receptor, as demonstrated by proliferation assays via ^3H thymidine incorporation after *ex vivo* stimulation with soluble CD40L, anti-IgM antibody or soluble CD40L with recombinant IL-4 (133). In a different model using CR1/2 KO mice, Molina *et al.* also demonstrated decreased antibody responses to immunization with SRBCs. Citing previous experiments with KLH and SRBCs suggesting that impairments in complement could be overcome

with high doses of immunogen, Molina and colleagues immunized mice with high and low doses of SRBCs. Although they found only a moderate decrease in IgM with both low and high doses of IgM, there was a dramatic impairment of IgG, specifically in IgG1 and IgG2b, indicating that a deficiency in complement receptor activation could not be overcome from simply a higher dose of antigen (134). Thus, from Fischer's and Ahearn's studies from the Carroll Lab, as well as confirmation by Molina *et al.,* it became apparent that disruption of either protein in the interaction between C3 and CR2 led to similar deficiencies in antibody development and germinal center formation, without affecting B cell signaling and proliferation.

Because earlier studies examining the disruption of CR2 or C3 in the development of humoral immunity focused on model antigens, Da Costa *et al.* and Ochsenbein *et al.* next wanted to investigate the role of complement in infections agents, specifically in the context of a viral infection. In a similar fashion to previous studies, Da Costa and colleagues infected mice with HSV and examined the role of complement in T cell proliferation, memory response and germinal center formation. Post-infection, C3 KO, C4 KO and CR1/2 KO mice were all unable to mount a robust antibody response compared to wild-type mice. Furthermore, as shown through immunofluorescence using the B cell marker B220 and the germinal center marker PNA, C3 KO and C4 KO mice possessed smaller germinal centers post-infection. However, T cell proliferation assays using ^{3}H thymidine incorporation after isolating lymph nodes and culturing in the presence of HSV lysate at different concentrations to prime T cells *ex vivo*, did not demonstrate any discernable difference between T cell activation among T cells isolated from C3 KO or CR1/2 KO mice (135). Similar to Da Costa *et al.* in examining the

response of C3 KO mice to viral infections, Ochsenbein *et al.* infected C3 KO mice with vesicular stomatitis virus (VSV) and found that C3 KO mice experienced increased mortality when infected with high doses of VSV. In terms of their antibody response, C3 KO mice also had decreased IgG titers after immunization with non-replicating viral antigen and interestingly, an early IgM response with high dose VSV. Contrary to other investigators in studying the antibody responses in C3 KO mice, Ochsenbein *et al*. actually found that C3 KO mice had a similar response to LCMV as wild-type mice. While Ochsenbein and colleagues did not further discuss why C3 can be critical in the setting of a humoral response to VSV or other viruses but not to LCMV. Another important and noteworthy aspect of the studies completed by Ochsenbein *et al.* was their depletion of $CD4^+$ T cells in C3 KO, C4 KO and CR1/2 KO mice, which is one of the only papers where the synergistic interaction of $CD4^+$ T cells with complement was examined. They found that even the IgM response of C3 KO, C4 KO, CR1/2 KO and wild-type mice to VSV are dependent on $CD4^+$ T cells, with a decreased IgM detectable by ELISA following depletion of $CD4^+$ T cells and exposure to VSV. Furthermore, C3 KO mice exhibit $CD4^+$ T cell dependence to poliomyelitis and vaccinia virus as well, where the mice developed IgM , but no subsequent IgG response following depletion of CD4 T cells and exposure to poliomyelitis or vaccinia virus. However, although C3 KO mice differed in their antibody responses to VSV and other viruses compared to wild-type mice, C3 KO, C4 KO and CR1/2 KO mice maintained serum IgG titers compared to wild-type mice when followed up to 150 days post-infection. Additionally, C3 KO, C4 KO and CR1/2 KO mice also maintained IgG titers to VSV in both the spleen and bone marrow, indicating that while complement receptors and complement components may

be important in the primary response to an infection, absence of complement receptors and components does not prevent the formation of long-lived plasma cells against VSV infection (136). Therefore, even in a majority of model systems utilizing viral antigens, such as HSV and VSV, complement receptors and components were shown, once again, to be critical in the formation of a primary antibody response post-infection.

While Da Costa and colleagues showed that C3 and CR2 are important in the humoral response to HSV, other investigators also sought to determine how the immune response in C3 KO and other complement component-deficient mice differed depending on the type of infection and route of administration. For example, Wessels *et al.* from the Carroll Lab infected C3 KO, C4 KO and wild-type mice with Group B Streptococcus (GBS), which has been implicated in neonatal meningitis. C3 KO and C4 KO mice infected with GBS experienced an increase in viable bacteria as measured by colony forming units, indicating that C3 and C4 are important in either bacterial lysis or clearance of the bacteria. Interestingly, C3 KO mice had more of a defect in decreasing their bacterial loads post-infection, suggesting that C3 may play more of a role in bacterial clearance or lysis compared to C4. Rather than just infecting C3 KO and C4 KO mice with GBS, Wessels *et al.* also used a more clinically translatable model of GBS infection in the setting of pregnancy, where it is commonly thought that mothers pass GBS to their infants as they pass through the birth canal, allowing GBS to colonize the upper respiratory tract of infants, leading to potential meningitis or other complications. After passively immunizing pregnant C3 KO and C4 KO mice with rabbit serum (from rabbits that were vaccinated with GBS type III polysaccharide-tetanus toxoid conjugate vaccine), pups were challenged with GBS. C3 KO pups from mothers immunized with

rabbit sera experienced increased mortality compared to C4 KO or wild-type pups, indicating more of a role for the classical pathway of complement in immunity against GBS (137). Contrary to Wessels *et al.* where GBS was administered systemically, Kerr *et al.* intranasally infected C3 KO mice with different strains of *Streptococcus pneumoniae* to further examine the role of C3 in an infection with gram-positive bacteria. Compared to wild-type mice intranasally infected with *S. pneumoniae,* C3 KO mice experienced increased bacterial loads in the lung, as measured in the lung tissue, bronchoalveolar lavage (to examine the cells in the interstitial space), as well as in the blood. Post-infection, C3 KO mice also displayed increased TNF and IL-6 cytokine expression in the lung homogenate, as well as in the bronchoalveolar lavage fluid. However, when Kerr *et al.* infected mice that were deficient in the C5a receptor, they found that C5a receptor, which binds the C5a anaphylaxotoxin to recruit neutrophils and other immune mediators, was not required for phagocyte recruitment to the lung region post-infection (138). Thus, both Wessels *et al.* and Kerr *et al.* demonstrated that deficiency of C3, in the context of a gram-positive bacterial infection, leads to increased bacterial loads and mortality post-infection.

In addition to examining whether complement receptors, specifically CR2, are required to mount a robust immune response, other investigators examined the mechanism behind CR2's connection with the germinal center and subsequent antibody development. In particular, Fischer *et al.* from the Carroll Lab crossed CR2-deficient mice with transgenic mice whose immunoglobulin heavy and light chains bind HEL with differing affinities: DEL with low affinity and TEL with high affinity, referred to as "DEL Ig" or "TEL Ig" mice. To examine the role of CR2 as a co-receptor for

complement-decorated antigen, splenic B cells from $CR2^{+/+}$ x DEL Ig mice or CR1/2 KO x DEL Ig B cells were adoptively transferred into wild-type mice immunized with DEL. Upon examination of the number of peripheral B cells from the transfer as well as the localization of B cells to the spleen, there was decreased splenic localization of peripheral B cells from wild-type mice that received CR1/2 KO x DEL Ig B cells, indicating that either anti-HEL antibody affinity or CR2 may affect the antibody response. To further differentiate whether antibody affinity to antigen or CR2 had a greater impact on B cell migration and maturation, $CR2^{+/+}$ x TEL Ig mice or CR1/2 KO x TEL Ig B cells were then adoptively transferred into wild-type mice primed with TEL, which resulted in decreased germinal center formation in TEL-primed recipients transferred with CR1/2 KO x TEL Ig B cells. Together, these results indicate that regardless of the immunoglobulin affinity for antigen, CR2 is critical for germinal center formation and that antigen affinity may allow for sufficient retention of B cells, but not formation of germinal centers (139). Consequently, from this paper and previous studies on the role of CR2, complement receptors were found to be important in germinal center formation, thus facilitating plasma cell development and antibody responses.

To further elucidate the mechanism behind CR2's involvement in humoral immunity to an infection, Croix *et al.* utilized Rag-2 deficient blastocyst complementation by injecting CR2-deficient embryonal stem cells into RAG2-deficient blastocysts in order generate mice deficient in CR2 specifically on hematopoetic cells with wild-type expression on FDCs. These chimeric mice were unable to mount a robust antibody response to KLH, further showing that B cell expression of CR2 is important for amplification of the antibody response to antigen (140). Furthermore, Fang *et al.*

hypothesized that the decreased antibody response seen in CR1/2 KO mice can be due to either decreased B cell stimulation or decreased deposition of antigen on the FDC surface leading to decreased B cell maturation. To differentiate between these two hypotheses, Fang *et al.* experimented with bone marrow reconstitution of CR1/2 KO mice, in which they injected *in vitro* pre-formed immune complexes (141) into chimeric CR1/2 KO mice reconstituted with wild-type bone marrow. Because FDCs are radioresistant and would not be replaced by donor bone marrow, the FDC phenotype was dependent upon the bone marrow recipient, whereas the B cell phenotype (whether B cells were CR2 negative or positive) was dependent on the donor phenotype, allowing for differentiation of the role of CR2's expression on B cells versus FDCs. CR1/2 KO mice reconstituted with wild-type bone marrow, meaning they had CR2-negative FDCs but CR2-positive B cells, mounted an initial antibody response to KLH or SRBCs but could not maintain it, leading to decreased IgG titers by day 14 and indicating that CR2 expression specifically on FDCs was critical to maintaining a robust humoral response. Furthermore, no antigen trapping occurred in CR1/2 KO reconstituted with wild-type bone marrow and these mice also had smaller but detectable germinal centers, suggesting that FDC expression of CR2 is critical for antigen trapping and formation of the germinal center. Comparatively, wild-type mice reconstituted with CR1/2 KO bone marrow formed germinal centers and mounted an antibody response compared to wild-type mice reconstituted with wild-type bone marrow, albeit the mice reconstituted with CR1/2 KO bone marrow had a decreased antibody response, demonstrating that CR2 expression on B cells is important for mounting a robust immune response but that an antibody response could still occur even in the absence of B cell CR2 expression (142). Thus, studies utilizing bone marrow

chimeras between CR1/2 KO mice and wild-type mice demonstrated that CR2-expression on FDCs is critical for germinal center formation, while CR2-expression on B cells is particularly important for mounting a robust antibody response to antigen.

Together these studies suggest that complement is fundamentally critical to the development of a robust antibody response, which has been demonstrated in a variety of settings, both traditional immunological models, as well as a range of viral and bacterial infections.

COMPLEMENT'S ROLE IN T CELL IMMUNITY

While complement's role in antibody development and humoral immunity has been studied for over 100 years, complement's involvement in T cell activation has only started to be characterized starting in the early 2000s. Here I will briefly discuss some key papers that built the foundation for what we know about complement's role in T cell activation.

To elucidate the role of complement and complement receptors in the context of T cell immunity, Kopf *et al.* described the course of influenza infection in C3 KO and CR1/2 KO mice. Compared to wild-type mice, C3 KO mice were more susceptible to influenza infection, with delayed viral clearance and increased viral titers. On the other hand, CR1/2 KO mice cleared the infection normally, indicating that C3 itself, and not its receptor on B cells or FDCs, was responsible for mediating influenza viral immunity. Kopf *et al.* also described that priming of T helper cells and cytotoxic T lymphocytes was also decreased in C3 KO mice compared to controls. Furthermore, the recruitment of virus specific $CD4^+$ and $CD8^+$ T cells to the lung was decreased, suggesting that C3 is

involved in the chemotaxis of T cells. Bronchoalveolar lavage (BAL) revealed that $CD4^+$ and $CD8^+$ T cells specifically in the BAL fluid had decreased expression of CD4 and CD8, in terms of surface staining. However, rather than the sheer number of T cells being decreased in the BAL fluid, Kopf *et al.* also found that BAL fluid $CD4^+$ T cell division was decreased compared to control mice in terms of ^3H thymidine incorporation. Additionally, after *in vitro* culture of $CD4^+$ T cells with dendritic cells and inactivated influenza, $CD4^+$ T cells from C3 KO mice produced less IFN-γ compared to $CD4^+$ T cells from control mice (143). Together these results suggest that complement may not only impact B cell immunity, but also T cell immunity in terms of T cell activation and proliferation.

As opposed to studying the involvement of complement in T cell immunity in terms of a viral infection, Drouin *et al.* utilized a model of asthma, where $C3aR^{-/-}$ mice, deficient in the receptor for the anaphylaxotoxin C3a, were challenged with *Aspergillus fumigates* and then studied for airway hyper-responsiveness and cytokine production. After challenge with *Aspergillus fumigates*, wild-type and $C3aR^{-/-}$ mice were ventilated and airway hyper-responsiveness was examined in terms of airway resistance to increasing doses of intravenous acetylcholine, a neurotransmitter that regulates bronchoconstriction and mucus production. $C3aR^{-/-}$ mice experienced decreased airway hyper-responsiveness, in that increased doses could be given to $C3aR^{-/-}$ mice before airway resistance occurred, compared to wild-type mice, indicating C3aR's involvement in the airway hyper-reactivity associated with asthma. Furthermore, following infection with *Aspergillus fumigates*, $C3aR^{-/-}$ mice had decreased recruitment of eosinophils and neutrophils in their bronchoalveolar fluid, as well as decreased mucin levels in the

bronchoalveolar fluid as well. Examination of cytokines in single cell suspensions of whole lung cells 24 hours past the last challenge with *Aspergillus fumigates* revealed decreased IL-5, IL-4 and IL-13 in C3aR$^{-/-}$ mice, which are the predominant Th2 cytokines believed to be involved in asthma pathogenesis (144). Therefore, although it was known that Th2 cytokines may be important in asthma pathogenesis, the association between the complement cascade, specifically C3aR, and the production of Th2 cytokine in a model of asthma was not as evident, further indicating complement's involvement in diseases previously thought to involve primarily T-cell mediated processes independent of complement.

Whereas Kopf, Drouin and colleagues studied the effect of hepatic-synthesized C3 on T cell responses, other investigators sought to understand how C3 synthesized in other cell types could affect T cell immunity. More specifically, Peng *et al.* found that dendritic cells produce C3, and therefore investigated how dendritic cell synthesis of C3 affected presentation to T cells. To study dendritic cell production of C3, they co-cultured dendritic cells from C3 KO mice, which are on a C57BL/6 background, with CD3$^+$, CD4$^+$ or CD8$^+$ T cells from a Balb/c background to examine allogeneic T cell responses. They found that dendritic cells from C3 KO mice had a reduced ability to stimulate alloreactive CD4$^+$ T cells, resulting in decreased production of IFN-γ. Likewise, dendritic cells from C3 KO mice on a Balb/c background had reduced ability to stimulate C57BL/6 T cells in culture. To determine the potential mechanism of the decreased alloreactive stimulation observed, C3 KO dendritic cells also showed increased expression of FoxP3 mRNA than wild-type dendritic cells (145). In addition to examining the T cell response in the absence of complement, Peng *et al.* also co-cultured dendritic cells from C57BL/6 mice

with Balb/c T cells and found production of IL-12 and IL-4, leading them to conclude that C3 promotes skewing towards a Th2 phenotype. Also examining C3 synthesis outside of hepatocytes and the effect on T cell stimulation was Yalcindag *et al.,* who examined C3 production from keratinocytes and peritoneal macrophages. When they sensitized C3 KO mice with an epicutaneous sensitization of ovalbumin (OVA) versus intraperitoneal immunization with OVA in alum, they found decreased eosinophil skin infiltration and decreased blood eosinophils in sensitized skin sites. They also found decreased expression of IL-4 and IL-5 in sensitized skin sites. Furthermore, splenocytes from C3 KO mice secreted less IL-4, IL-5, IL-13 and IFN-γ in response to OVA stimulation or $CD3^+$ stimulation, which was also observed after intraperitoneal injection of OVA. However, the deficits in cytokine production could be rescued by supplementation of purified human C3 *in vitro*, suggesting that C3 production by keratinocytes and peritoneal macrophages could stimulate both a Th1 and Th2 phenotype (86). Consequently, these studies suggested that cells outside of the liver could synthesize C3 and that these extra-hepatic sources of C3 could lead to T cell stimulation in the setting of an alloreactive T cell response or skin allergy model.

In addition to studying the effects of extra-hepatic C3 on T cell immunity, other investigators sought to determine how C3 and its cleavage products, such as C3a and C5a, could impact T cell activation. To address the mechanism and impact of C3a and C5a on T cell activation, Strainic *et al.* incubated of T cells from OTII mice with APCs from WT, $C3aR^-$, $C5aR^-$ or $C3aR^-$ $C5aR^-$ double knockout mice. Following stimulation with CD3 and CD28, only OTII T cells incubated with antigen-presenting cells (APCs) from wild-type mice upregulated C5 and C5aR mRNA post-activation, leading to CD40L

mRNA expression, followed by IL-12 mRNA expression. Additionally, IL-12 mRNA up regulation by dendritic cells preceded OVA-specific OTII T cells synthesis of IFN-γ. In contrast, when OTII T cells were incubated with C3aR$^-$, C5aR$^-$ or C3aR$^-$ C5aR$^-$ double knockout APCs, there was decreased production of IFN-γ *in vitro* (146), suggesting that C3a and C5a receptors play a more direct role in T cell activation than previously thought. Furthermore, it was more recently shown that the interaction of another component of the complement cascade, C3b, with the complement receptor CD46 induced expression of both an amino acid transporter LAT1, as well as a glucose transporter GLUT1 on T cells. CD46 was also shown to increase mTOR phosphorylation and mTORC1 complex assembly, further contributing to maintenance of T cell activation (147). Taken together, these studies demonstrate complement's role in T cell activation, metabolism and survival.

To study the role of complement receptors in an infection, C3aR$^-$, C5aR$^-$ or C3aR$^-$ C5aR$^-$ double knockout mice were infected with *Toxoplasmosis gondii* and experienced increased mortality, as well as decreased IL-12 production and IFN-γ producing cells at 10 days post-infection compared to wild-type mice. Furthermore, to address whether C3a and C5a receptors affected APC activation and co-stimulation, Strainic *et al.* found that APCs from C3aR$^-$, C5aR$^-$ or C3aR$^-$ C5aR$^-$ double knockout mice did not upregulate CD80/CD86 nor CD40 and that T cells from C3aR$^-$, C5aR$^-$ or C3aR$^-$ C5aR$^-$ double knockout mice did not upregulate CD40L and CD28 compared to wild-type mice (146), suggesting that complement anaphylaxotoxin receptors are not only important in controlling a bacterial infection, but also in APC co-stimulation and subsequent T cell activation.

Although it had been shown that C3 circulates extracellularly in the sera possibly due to synthesis by hepatocytes, C3 is also expressed intracellularly in T cells. Liszewski *et al*. described that intracellular C3a is important in T cell homeostasis and survival. Cathepsin L was shown to process intracellular C3 into C3a and C3b, after which C3a is shuttled to the surface upon T cell activation, thereby inducing cytokine production. Upon addition of a cathepsin L inhibitor *in vitro*, the viability of T cells decreased, indicating that intracellular C3a is important for T cell survival. Furthermore, the mechanism of C3a-mediated T cell activation was elicited when addition of C3a to T cells in culture resulted in an increase in phosphorylated mTOR expression, which was inhibited by addition of a cathepsin L inhibitor (148-150). As it was more recently demonstrated that cells establish intracellular C3 stores from uptake of extracellular C3(H_2O) through some yet unidentified receptors (151), these results demonstrate that the intracellular involvement of C3 in the activation of $CD4^+$ T cells requires a complex uptake, recycling and storage mechanism.

In conclusion, as we have seen in the previous studies, both intracellular and extracellular complement are important in T cell activation and viability with a variety of implications, ranging from cytokine stimulation to co-stimulation upregulation.

CONCLUDING REMARKS: THE COMPLEMENT SYSTEM IN IMMUNITY

From the early studies involving SRBCs with guinea pig serum to recent developments in our understanding of complement's role in T cell activation, the field of complement biology evolved over the last 100 years. However, much about the

complement cascade remains to be understood, specifically how complement may be involved in the humoral and T cell responses to RBC antigens.

Although complement fixation on the RBC has been a staple of complement biology since its earliest days, complement's involvement in the immunity to RBC antigens in particular has been largely absent from the complement literature. Further study into how complement components are multi-faceted in nature will, without doubt, provide greater insight into how complement is involved in many aspects of the immune system.

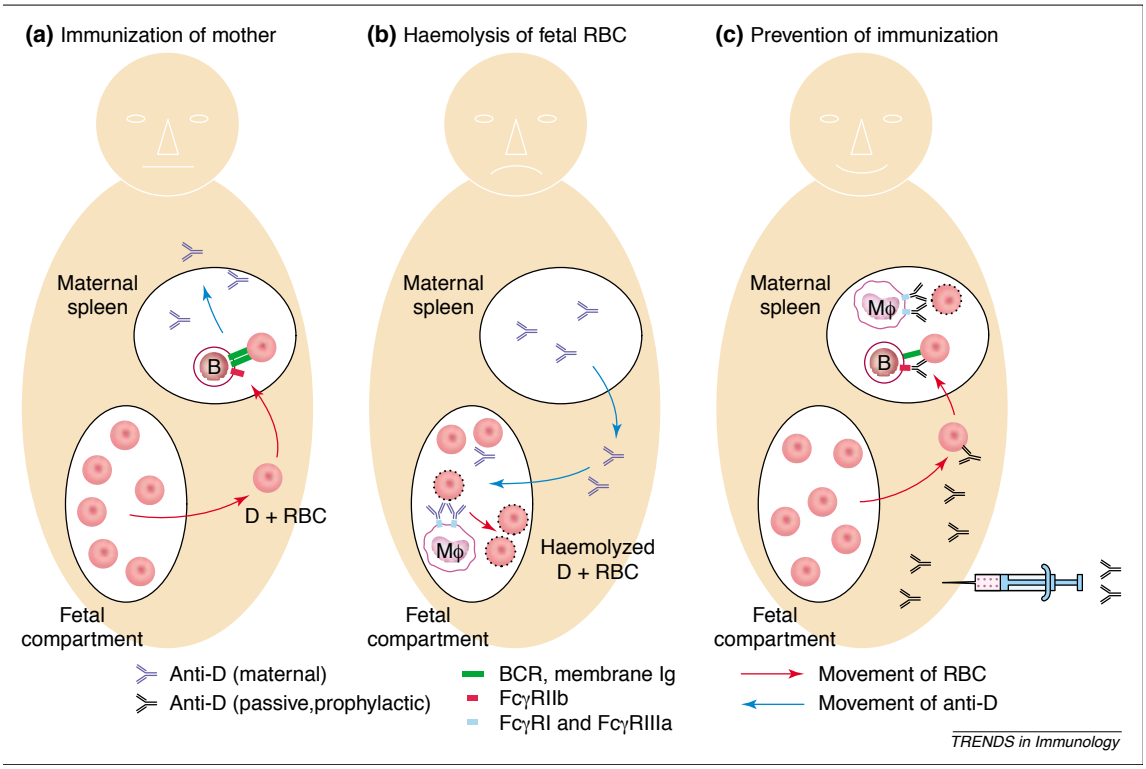

Image courtesy of Kumpel *et al. Trends in Immunology*. 2001.

Figure 1: During fetomaternal hemorrhage, (a) RhD^- mothers exposed to RhD^+ fetal RBCs can develop alloantibodies against the RhD RBC antigen. (b) These anti-RhD alloantibodies can cross the placenta during subsequent pregnancies, causing HDFN. (c) Administration of RhIg, consisting of pooled serum from RhD^- donors who have become sensitized to RhD^+ RBCs through pregnancy or transfusion, can prevent the development of anti-RhD alloantibodies, though its exact mechanism is not well-understood (152).

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Chapter 2: Antibody-mediated immunosuppression can result from RBC antigen loss independent of Fcγ receptors in mice

Abstract

Background: Anti-RhD antibody administration can prevent *de novo* anti-RhD alloantibody formation following RhD^+ red blood cell (RBC) exposure, termed antibody-mediated immunosuppression (AMIS). Recent studies suggest that AMIS may occur through target antigen alterations, known as antigen modulation. However, studies suggest that AMIS may occur independent of antigen modulation. In particular, AMIS to RBCs that transgenically express the fusion hen egg lysozyme-ovalbumin-Duffy (HOD) antigen have been shown to occur independent of activating Fcγ receptors (FcγRs) thought to be required for antigen modulation. Therefore, we sought to determine the mechanism behind AMIS following HOD RBC exposure.

Study Design and Methods: Following transfer of HOD RBCs into wild-type or FcγR-chain knockout recipients in the presence or absence of anti-HEL monoclonal antibodies individually or in combination, HOD antigen levels and anti-HOD antibody formation were examined.

Results: Our results demonstrate that anti-HEL antibodies individually or in combination suppressed anti-HOD IgM, which correlated with the rate of detectable decrease in HEL on HOD RBCs. Furthermore, exposure to anti-HEL antibodies alone or in combination equally suppressed anti-HOD IgG formation. Unexpectedly, combination or individual anti-HEL antibodies induced AMIS and antigen modulation in an FcγR-independent manner. Pre-exposure of HOD RBCs to anti-HEL antibodies reduced antigen levels and suppressed anti-HOD antibody formation following HOD RBC exposure.

Conclusion: These results suggest that antibody-mediated antigen modulation may reflect a mechanism of AMIS that can occur independent of activating FcγRs and may provide a surrogate to identify antibodies capable of inducing AMIS against different RBC alloantigens.

Introduction

Alloantibodies to distinct red blood cell (RBC) antigens developed following transfusion or pregnancy can lead to hemolytic disease of the fetus and newborn (HDFN) (1-4). Currently, the only commercially available prophylaxis that can actively prevent anti-RBC alloantibody development following alloantigen exposure is Rh immune globulin (RhIg) (5-10). RhIg, anti-RhD polyclonal antibody generated from pooled donor serum administered to prevent *de novo* anti-RhD antibody development, is the most commonly employed example of antibody-mediated immunosuppression (AMIS). As the availability of RhIg donors is limited and the only available immunoprophylaxis is against RhD, there is a critical need to develop monoclonal antibody alternatives to RhD and immunoprophylaxis to other RBC alloantigens. As different monoclonal antibodies to the RhD antigen can have differential immunological outcomes (11-20), understanding the mechanism regarding how monoclonal antibodies induce AMIS can facilitate the development and accurate assessment of monoclonal antibodies against RhD and other RBC alloantigens.

While the mechanisms responsible for RhIg-induced AMIS remain unknown, several hypotheses have been suggested, including rapid clearance and/or steric hindrance of RhD^+ RBCs to prevent B cell recognition or direct immune modulation (21-23). However, as RBC clearance rates do not necessarily correspond to the ability of an antibody to induce AMIS (23) and several studies suggest that steric hindrance or inhibition of the immune response through inhibitory Fcγ receptors (FcγRs) may not play a role in AMIS (24-28), alternative mechanisms may be responsible. Recent studies using the KEL model system demonstrate that AMIS to the KEL RBC alloantigen

correlates with antigen modulation (29, 30), a phenomenon referring to antibody-induced loss of the target RBC antigen. In this setting, anti-KEL antibodies induce complement fixation and subsequent antigen modulation, which was found to be dependent on both complement and activating FcγRs (31-36). Similarly, previous studies demonstrate that FcγRs are also required for anti-HEL (hen egg lysozyme) induced antigen loss following engagement of HEL antigen expressed in a membrane-bound form on RBCs (mHEL RBCs) (36). However, when HEL is expressed along with ovalbumin and human Duffy as a fusion protein on RBCs (HOD RBCs), AMIS to HOD occurs independent of FcγRs (28), suggesting that anti-HEL-induced AMIS following HOD RBC exposure may occur independent of antigen modulation. These collective data suggest that while antigen modulation may mediate anti-KEL-induced AMIS, anti-HEL antibodies likely induce AMIS to the HOD antigen through an alternative mechanism. Furthermore, while previous studies have separately examined monoclonal antibody-mediated AMIS to the HOD alloantigen and the role of FcγRs in antigen modulation of HEL on mHEL RBCs (27, 36, 37), no studies have formally examined whether anti-HEL monoclonal antibodies mediate antigen modulation to the HOD antigen. Therefore, we sought to determine whether AMIS to HOD RBCs occurs via monoclonal antibody-induced antigen modulation.

To study this, we utilized the previously established HOD RBC model to define the impact of potential changes in surface RBC antigen as a mechanism responsible for AMIS (27, 28, 38, 39). Our results demonstrate that anti-HEL monoclonal antibodies individually or in combination inhibit the downstream antibody response to HOD RBCs, in support of previous findings (27). Anti-HEL-induced AMIS not only correlated with a

decrease in the level of detectable HEL antigen on the RBC surface, but both antigen modulation and AMIS occurred independent of activating FcγR function. Together, these data suggest that monoclonal antibodies may inhibit antibody responses to some RBC alloantigens through FcγR-independent modulation of the target antigen.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from Charles River and Fcer1g (FcγR KO) mice deficient in the common gamma chain were purchased from Taconic Laboratories. HOD transgenic mice were generated as previously described (39). Emory Division of Animal Resources and Husbandry bred and housed the mice used in these experiments. Male and female mice were used at ages 8 to 12 weeks. The experimental protocols and animal procedures performed in these studies were approved by the Emory University Institutional Animal Care and Use Committee.

RBC labeling and transfusion

HOD^+ and HOD^- control RBCs were collected and labeled as previously described (37, 38, 40, 41). Each recipient received approximately 10^7 HOD^+ RBCs (1:1 with HOD^- RBCs) sensitized with 2.5 □g/mL IgG1 anti-HEL monoclonal antibody (clone: 2F4), 2.5 □g/mL IgG1 anti-HEL monoclonal antibody (clone: 4B7), or 1.25 □g/mL of each anti-HEL monoclonal antibody 2F4 and 4B7 in combination prior to transfusion, with PBS alone as a control, as described previously (27, 28).

RBC staining and antibody identification by flow cytometry

Post-transfusion, mice were sampled at 10 minutes, 2 hours, days 1, 2, 3 and 5 post-transfusion. For assessing detectable HEL antigen, RBCs were stained with 1:500 anti-HEL antibodies 2F4 and 4B7, followed by anti-mouse IgG (Jackson Immunoresearch), as done previously.(36, 37, 42) For the direct antiglobulin test (DAT), RBCs were stained

with anti-mouse IgG. For complement component 3 (C3) detection, RBCs were stained with anti-mouse C3 biotin (Cedarlane), followed by streptavidin (BD) (40, 41). For detection of Duffy, RBCs were stained with 1:500 anti-Duffy monoclonal antibody, MIMA-29, followed by anti-mouse IgG. For detection of Ter119, RBCs were stained with Ter119 (BD), as done previously (40). To assess anti-HOD IgM and IgG antibody development, sera was flow cross-matched, as previously described (29, 30, 43-46). All antibodies were used at 1:100 in FACS buffer (2% bovine serum albumin in phosphate buffer saline) unless otherwise noted, as done previously (29, 36, 37, 40-42, 47, 48).

Western blot analysis

RBCs isolated from each recipient group 2 days post-immunization with anti-HEL monoclonal antibodies were lysed in 5 mmol/L sodium phosphate (pH 7.5) with protease inhibitor cocktail (Sigma Roche) at 4°C and centrifuged at 14,000 rpm, as outlined previously (29, 48). RBC ghosts were then subjected to western blot analysis with rabbit polyclonal anti-HEL antibody (Abcam, ab391, 1:5,000) and detection with goat anti-rabbit HRP (Abcam, ab205718 1:10,000). Mouse monoclonal anti-GAPDH (clone: GA1R, 1:1,000), followed by goat anti-mouse IgG1 HRP (Bethyl Laboratories, 1:10,000) was applied following stripping membranes with 25 mM glycine-HCl with 1% SDS, pH 2. All membranes were developed using HyGlo ECL (Denville Scientific Inc.).

Protease treatment

Following sensitization with anti-HEL monoclonals individually or in combination, HOD RBCs were incubated with decreasing concentrations of Pronase (Sigma) starting from

2.5 □g/mL for 30 minutes at 37°C. Following protease treatment, RBCs were stained for HEL, Duffy and Ter119 antigens as outlined above.

Biotinylation

HOD mice were intravenously injected with 1 mg of EZ-Link Sulfo-NHS-Biotin (ThermoFisher Scientific). RBCs were stained with streptavidin (BD) and evaluated for the levels of detectable HEL, Duffy and Ter119 as described (40).

Statistics

Flow cytometry data was analyzed in FlowJo (version 9.9.6) and statistical analysis was performed in GraphPad Prism (version 7). Three or more groups were compared by One-way ANOVA with multiple comparisons by Tukey's test. For analysis of RBC survival and changes in detectable antigen following protease treatment, Two-way ANOVA with Dunnett's or Tukey's multiple comparisons test was utilized, respectively. $p<.05$ was the cut off for significance.

Results

Anti-HEL monoclonal antibodies decrease antibody formation and detectable antigen

We utilized a previously established model of AMIS wherein B6 recipients are exposed to HOD RBCs in the presence or absence of saturating concentrations of 2F4 and 4B7 anti-HEL monoclonal antibodies individually or in combination (Figure 1A), each of which recognize distinct HEL epitopes (Figure 1B) (27, 42). Consistent with previous studies in which anti-HOD IgM is detectable 3-5 days following transfusion of HOD RBCs and class switching to IgG occurring between days 7-14 post-transfusion (43, 44, 49, 50), exposure to HOD RBCs resulted in an anti-HOD IgM and IgG response, with no development of anti-HOD antibodies in HOD mice exposed to HOD RBCs (Figure 1B). However, when HOD RBCs were transfused in the presence of anti-HEL antibodies, individually or in combination, the anti-HOD IgM response was blunted on day 5 post-transfusion (Figure 1B). Furthermore, the anti-HOD IgG response was also suppressed in the presence of a combination or individual monoclonal antibodies (Figure 1B). To determine whether antibody engagement differed over time, mice were exposed to labeled HOD RBCs and RBCs were analyzed by a flow cytometry-based direct antiglobulin test (DAT) (Figure 2A-B). The DAT revealed that IgG could be detected directly on HOD RBCs in the presence of anti-HEL antibodies at 10 minutes and 2 hours post-transfusion (Figure 2C-D). However, exposure to a combination of anti-HEL monoclonal antibodies resulted in a more rapid decrease in DAT levels than anti-HEL antibodies individually, with DAT levels decreasing through 5 days post-transfusion (Figure 2E-H).

While polyclonal antibodies have been shown to induce modulation of the HEL antigen on mHEL RBCs (36, 41), monoclonal antibodies alone were not found to induce alterations to the target antigen (42), suggesting that the loss of detectable antibody on the HOD RBC surface over time may reflect antibody dissociation. To test this, the level of detectable antigen was examined over time (Figure 3A). By 10 minutes and 2 hours post-transfusion (Figure 3B-C), the level of detectable HEL antigen was decreased in B6 mice exposed to HOD RBCs in the presence of individual or a combination of anti-HEL antibodies. Similar to the antibodies detected on the cell surface (Figure 2), the level of HEL antigen following exposure to a combination of monoclonal antibodies was nearly undetectable. The level of HEL antigen on HOD RBCs in B6 mice exposed to anti-HEL monoclonal antibodies individually decreased over time when compared to HOD RBCs transfused into HOD mice (Figure 3B-G). Loss of detectable antigen did not appear to reflect anti-HEL induced complement masking of the HEL antigen or RBC clearance, as no complement was detected on HOD RBCs when evaluated in parallel nor clearance occurred regardless of anti-HEL antibody exposure (Figure 3H-I), consistent with previous results (36, 42). Importantly, although the level of HEL antigen decreased by day 5 post-transfusion in B6 mice exposed to HOD RBCs in the absence of anti-HEL monoclonal antibodies, this HEL antigen decrease paralleled anti-HOD alloantibody development in the serum (Figure 1) (43, 44, 49, 50), suggesting that development of *de novo* anti-HEL antibodies in B6 mice exposed to HOD RBCs may also induce changes in the level of detectable HEL antigen over time.

AMIS can occur independently of activating FcγR function

Given that our findings thus far indicate that monoclonal antibody-induced AMIS to HOD RBCs may occur as a result of changes in the level of detectable HEL antigen in B6 mice and that AMIS induced by antibody-mediated antigen modulation of the HEL antigen on mHEL RBCs occurs through an FcγR-mediated process (36, 40), we tested the role of activating FcγRs in anti-HEL monoclonal antibody-induced modulation of the HEL antigen on HOD RBCs. To investigate this, recipients deficient in activating FcγRs (FcγR KO) were exposed to labeled HOD RBCs in the presence or absence of anti-HEL monoclonal antibodies, individually or in combination. In FcγR KO mice, HOD RBCs in the presence of anti-HEL monoclonal antibodies individually did not experience an initial decrease in their level of detectable HEL antigen at 10 minutes post-transfusion (Figure 4A), but began to display decreases in detectable antigen 2 hours post transfusion (Figure 4B). However, by day 1 post-transfusion, HEL antigen levels on HOD RBCs in FcγR KO mice exposed to individual monoclonal antibodies began to decline over time (Figure 4C-F).

Similar to the decrease in detectable HEL antigen observed in B6 mice, the HEL antigen level decreases in FcγR KO recipients exposed to HOD RBCs with anti-HEL monoclonal antibodies was also found to correlate with decreases in detectable antibody on the RBC surface. By 10 minutes post-transfusion, IgG antibody was detectable on the HOD RBC surface in FcγR KO mice exposed to HOD RBCs in the presence of anti-HEL monoclonal antibodies, individually or in combination (Figure 5A). However, from 2 hours through day 5 post-transfusion, the level of IgG antibody deposited on the HOD RBC surface decreased in FcγR KO mice that received HOD RBCs in the presence of anti-HEL monoclonal antibodies, individually or in combination (Figure 5B-F). This

decrease in bound IgG on the HOD RBC surface paralleled the decrease in detectable HEL antigen (Figure 4). Given the ability of anti-HEL monoclonal antibodies to decrease the level of detectable HEL antigen in B6 and FcγR KO mice, and as the decrease in antigen level correlated with AMIS in B6 mice (Figures 1-4), we tested whether AMIS likewise occurred in FcγR KO mice. Exposure of FcγR KO mice to HOD RBCs in the absence of anti-HEL monoclonal antibodies resulted in development of anti-HOD IgM and IgG at days 5 and 14 post-transfusion, respectively. However, exposure of FcγR KO mice to HOD RBCs in the presence of anti-HEL monoclonal antibodies resulted in equivalent inhibition of anti-HOD IgM and IgG antibody formation (Figure 6A). Similar to HOD RBC transfusion into B6 mice, anti-HEL monoclonal antibodies failed to fix detectable complement or alter HOD RBC clearance in FcγR KO mice (Figure 6B-C).

Re-transfusion of antigen-modulated HOD RBCs affects subsequent anti-HOD antibody response

The dampened anti-HOD IgM response observed following exposure to anti-HEL monoclonal antibodies individually or in combination correlated with the decrease in detectable HEL antigen in B6 mice exposed to HOD RBCs in the presence of anti-HEL monoclonal antibodies, which may reflect early alterations in the availability of HEL antigen observed following exposure to anti-HEL antibodies individually or in combination. Homozygous and heterozygous HOD mice exhibit no detectable differences in HEL antigen expression (Fig. 7A), precluding direct examination of the potential impact of antigen density in the absence of antibody-induced changes in antigen levels. Therefore, to more directly test this, B6 mice were first exposed to HOD RBCs in the

presence or absence of anti-HEL monoclonal antibodies to reach varying levels of antigen reduction. Using this approach, HOD RBCs were followed for changes in the level of detectable HEL antigen until a 50% or 90% reduction in HEL antigen on RBCs in the presence of anti-HEL monoclonal antibodies individually or in combination occurred (Figure 7B), respectively, to approximate the level of detectable antigen observed on HOD RBCs 1 day following antibody exposure (Figure 3D). RBCs were then harvested and re-transfused into naïve B6 recipients, followed by evaluation of the detectable HEL antigen over time to determine whether the HEL antigen further decreased in re-transfused mice. At 10 minutes and day 1 post-transfusion, the level of detectable HEL antigen on re-transfused HOD RBCs that were previously exposed to individual anti-HEL monoclonal antibodies remained stable (Figure 7C-D). However, by day 3 after re-transfusion, the level of detectable HEL antigen decreased on the HOD RBCs that previously circulated in the presence of anti-HEL monoclonal antibodies individually (Figure 7E). Furthermore, the level of detectable HEL antigen on HOD RBCs that were exposed to a combination of anti-HEL monoclonal antibodies remained low from 10 minutes through day 5 after re-transfusion (Figure 7C-F). Re-transfusion of HOD RBCs with decreased antigen due to prior exposure to anti-HEL monoclonal antibodies individually or in combination resulted in an equally dampened anti-HOD antibody responses (Figure 7G). The similar ability of individual or combinations of anti-HEL antibodies to induce AMIS does not appear to reflect differences in HOD RBC clearance following re-transfusion, as no change in HOD RBC clearance was observed following re-transfusion (Figure 7H).

HEL antigen decreases as HOD RBCs age

Given the ability of monoclonal antibodies to impact HEL antigen levels following HOD RBC exposure in FcγR KO mice, it remained possible that anti-HEL antibodies simply enhance an existing process of antigen removal on HOD RBCs. This is especially relevant when considering that antigens are not re-synthesized on mature RBCs, and therefore certain RBC antigens may be more susceptible to degradation over time. Consistent with this, HEL antigen levels were consistently lower than the Duffy antigen on RBCs harvested from HOD mice (Figure 8A), suggesting that HEL may be uniquely sensitive to degradation over time. To initially determine whether the HEL antigen may be more sensitive to proteolytic cleavage, HOD RBCs were incubated with a range of protease concentrations (Figure 8B). HEL antigen displayed more sensitivity toward proteolytic cleavage than Duffy or Ter119 antigens, which failed to experience similar changes in levels under the same conditions (Figure 8C-E). However, inclusion of anti-HEL antibodies failed to alter the differential sensitivity of HEL to proteolytic cleavage (Figure 8B-E). As a result, we next sought to simply determine whether HEL is selectively lost over time on HOD RBCs as they age *in vivo* in the absence of anti-HEL antibodies. To accomplish this, HOD RBC donor mice were biotinylated, followed by tracking HOD RBCs over time (Figure 8F). While biotinylation itself failed to impact the level of detectable HEL, Duffy or Ter119, by 14 days post-biotinylation, the level of HEL decreased. In contrast, similar changes failed to occur in Duffy or Ter119 when evaluated in parallel (Figure 8G-I). Despite the inability of anti-HEL antibodies to impact HEL antigen sensitivity to proteolytic cleavage *in vitro*, we next sought to determine whether selective loss of HEL likewise occurs following antibody-mediated HEL

antigen-removal (Figure 8J). HEL antigen on HOD RBCs experienced a 50% or 90% decrease in the presence of anti-HEL monoclonal antibodies, individually or in combination, respectively (Figure 8K) with no detectable change in Duffy antigen (Figure 8L). Despite the absence of detectable complement fixation on the RBC surface (Figure 3H), it remained possible that antibody fragments may mask the HEL antigen, resulting in apparent HEL antigen loss. To test this, western blot analysis was performed. Using this approach, HEL antigen loss was observed following exposure to anti-HEL monoclonal antibodies individually or in combination (Figure 8M). Taken together, these results suggest that anti-HEL antibodies may accelerate an existing process of HEL antigen removal.

Discussion

In this study, anti-HEL monoclonal antibodies were not only found to correlate with a dampened IgM antibody response to the HOD RBC antigen, but also a decreased level of detectable antigen itself. These findings suggest that monoclonal antibodies may induce AMIS by decreasing detectable HEL antigen levels below the threshold necessary to activate HEL-reactive B cells. B cell activation typically requires not only a threshold concentration of antigen, but also a threshold of receptor stimulation (51-56). Consistent with this, lowering the level of detectable HEL antigen by 50% resulted in an equivalent degree of IgM suppression. As no difference in the anti-HOD IgG response was observed whether HOD RBCs were transfused in the presence of anti-HEL antibodies alone or in combination, the similar level of IgG suppression suggests that by the time class switching occurs, HEL antigen levels may have dropped below the threshold needed to efficiently induce anti-HEL IgG. As suppression of IgG alloantibody formation is the primary target of immunoprophylaxis, these results suggest that monoclonal IgG antibodies that initially only partially induce antigen loss may be sufficient for equivalent inhibition of IgG formation.

As the consequence of antibody-antigen interactions can differ (28, 29, 36-38, 40-42, 47, 48, 57), the requirements of AMIS for a particular antigen may likewise uniquely depend on the target antigen. For example, the reduced efficacy of monoclonal antibodies to induce AMIS toward RhD may in part reflect a need for multiple antibodies to induce antigen modulation,(24, 58) as demonstrated for HEL antigen expression on mHEL RBCs (42). Although recent studies demonstrate that anti-RhD antibodies can induce antigen modulation clinically (59), it is certainly possible that for RhD and possibly other

antigens, a combination of RBC clearance and antigen modulation collectively contribute to AMIS. As anti-RhD represents the only clinically available AMIS intervention, understanding potential differences in the outcome of antibody engagement of a variety of RBC antigens is important in understanding both common and distinct features of antibody-antigen interactions that may result in AMIS. Such information will likely prove vital for rational approaches to be developed to target other antigen for which no immunoprophylaxis currently exists.

In addition to a potential threshold of detectable HEL antigen necessary to induce a productive anti-HOD antibody response, the rate at which the HEL antigen is modulated may likewise impact the development of anti-HOD antibodies. Given that HOD RBCs in the presence of anti-HEL monoclonal antibodies undergo a more rapid decrease in the level of detectable HEL antigen than HOD RBCs transfused into B6 recipients in the absence of anti-HEL monoclonal antibodies, the removal of antigen at early time points may prevent sufficient detection and therefore response to HOD at these earlier time points. Actual differences or trends toward reduced ability of individual monoclonal antibodies to suppress anti-HOD IgM may likewise reflect distinct windows wherein antigen substrate is needed for anti-HOD IgM antibody formation in contrast to the possibility of slightly later antigen requirements for IgG antibody formation. While the ability of anti-HEL antibody to modulate antigen suggest that such thresholds exist, the exact threshold requirements for productive antibody formation remain unknown. Unfortunately, as antibody provided the only mechanism of selectively removing HEL from HOD RBCs, we cannot rule out the potential impact of antibody remaining on the cell surface on antibody formation. While proteolytic cleavage allowed HEL to be

selectively removed *in vitro*, many proteins are selectively sensitive to protease (60), which could impact RBC circulatory half-life, interactions with immune cells or other RBC changes that may impact the overall immune response to HOD, precluding the use of this approach to examine the potential impact of HEL antigen levels on anti-HOD antibody formation. HOD founders that express different levels of HOD, as recently described for KEL (46), will ultimately be needed to begin defining potential antigen threshold requirements for a productive immune response against the HOD antigen.

While antigen removal still occurred in FcγR KO recipients, the rate of antigen modulation was reduced compared to B6 mice, suggesting that while antigen modulation and AMIS can occur independent of FcγRs, these receptors may contribute to early antigen removal. Consistent with this, previous studies indicate that FcγRs can facilitate antigen removal (40, 41). However, given that previous studies have refuted the involvement of both activating and inhibitory FcγRs in polyclonal antibody-mediated AMIS to HOD RBCs (28), and our study shows that the anti-HEL monoclonal antibodies do not fix complement nor are activating FcγRs required for AMIS or antigen modulation to occur, these studies therefore suggest a novel mechanism of antigen removal distinct from those previously described (29, 41). Similarly, while anti-HEL monoclonal antibodies individually or in combination induced AMIS equivalently in FcγR KO mice for both IgM and IgG, unimmunized FcγR KO mice may potentially develop a decreased antibody response to HOD RBCs compared to B6 mice, indicating that FcγRs may be involved in other aspects of anti-HOD immunity (61-65). As a result, while FcγRs are dispensable for AMIS in this model, they may impact anti-HOD antibody formation more generally.

As antigen is removed on HOD RBCs as RBCs age over time, despite no difference in HEL antigen levels *in vitro* following incubation of HOD RBCs with anti-HEL antibodies in the presence or absence of proteases, endothelial-derived or other organ resident proteases, in addition to shear forces, may cumulatively impact HEL antigen levels following antibody engagement *in vivo*. Given that ligand has been shown to activate platelets via proteolytic cleavage of cell surface proteins in a shear-dependent manner (66), it is possible that shear forces may potentially work synergistically with antibody to alter HOD cleavage site accessibility. In this setting, non-classical type II Fc receptors outside the type I FcγR family, including CD23 and possible other lectin receptors (67), may engage IgG, facilitating shear-dependent forces as antibody is tethered to the RBC through the HEL antigen. Similar mechanisms may be involved in the apparent ability of the endogenous immune response to likewise impact HEL antigen levels in the absence of anti-HEL monoclonal antibodies. However, it is important to note that increased sensitivity of HEL to proteolytic cleavage *in vitro* may not reflect antibody-induced loss of the HEL antigen *in vivo,* which may instead represent an entirely different process. Distinct mechanisms of antigen modulation may play unique roles in different antigen systems, each of which may be differentially sensitive to variables such as saturation, involvement of antibody effector systems and level of antibody antigen engagement required. Although antibody-induced antigen loss has been documented in various settings in both animal models and humans (30, 40, 48, 59, 68, 69), the underlying mechanisms whereby these processes occur remain to be determined.

As with all experimental models, limitations must be considered. In this study, we examine one particular RBC antigen in combination with two anti-HEL monoclonal

antibodies previously validated to induce AMIS in this model system (27). Therefore, while both anti-HEL monoclonal antibodies tested induced antigen modulation and recent studies demonstrate that monoclonal antibodies that target RBC antigens can induce antigen loss in patients (48, 59), whether antigen modulation is a general phenomenon that occurs following antibody engagement of other RBC alloantigens remains to be determined. Furthermore, despite lack of analysis of Duffy antigen by western blot analysis, loss of the HEL antigen by western blot analysis, coupled with similar loss of HEL detection by flow cytometric analysis in the absence of similar changes in Duffy antigen levels, suggests that anti-HEL antibodies induce selective antigen removal. While pre-clinical models possess limitations, these models allow us to investigate potential molecular mechanisms whereby general principles of AMIS may occur. As the KEL and HOD models are the only systems published to date that render themselves to AMIS studies, these findings suggest that antigen modulation may play a role in AMIS. However, whether this approach actually prevents alloimmunization against other RBC alloantigens remains to be tested. Using this and related model systems may therefore provide important insight when seeking to answer questions in transfusion medicine surrounding the development of antibodies capable of inducing AMIS.

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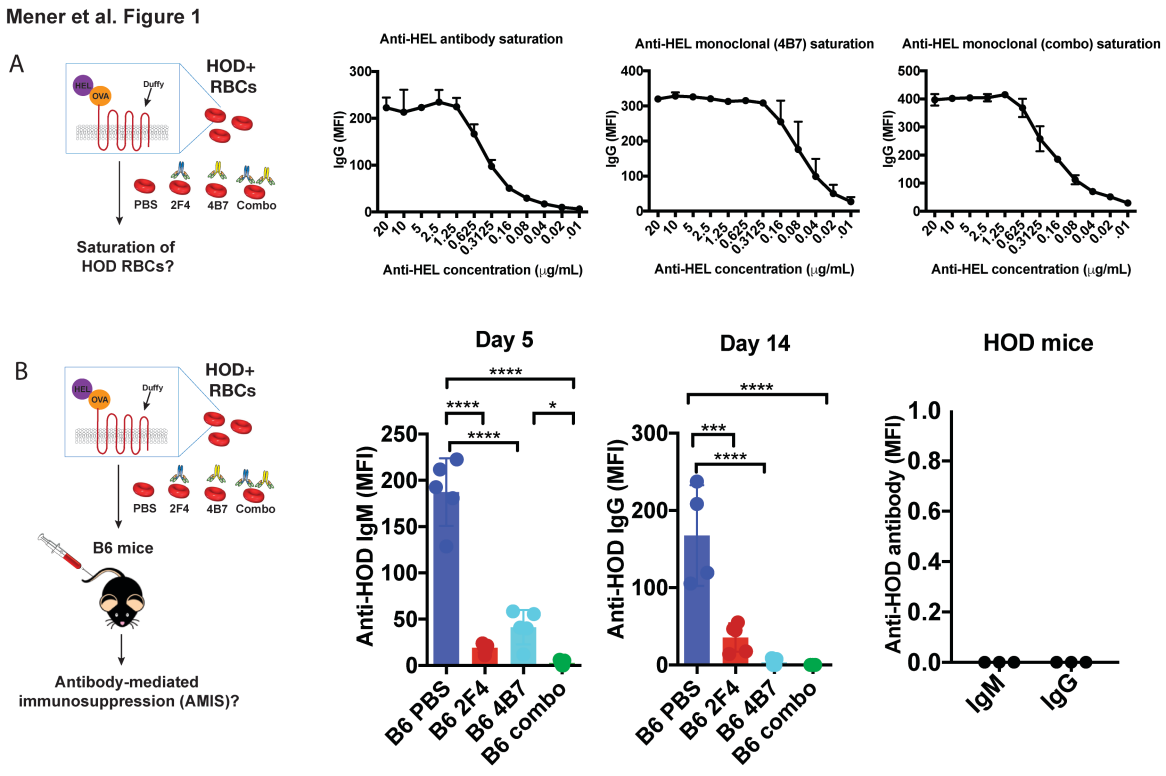


Figure 1: Anti-HEL monoclonal antibodies saturate HOD RBCs and dampen anti-HOD antibody response. (A) Antibody saturation of HOD RBCs individually or in combination was assessed by flow cytometry. (B) Following exposure of B6 mice to HOD RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 (combo)) or absence (PBS) of anti-HEL monoclonal antibodies, serum was assessed for the presence of anti-HOD IgM on Day 5 and anti-HOD IgG on Day 14 post-transfusion by flow cross-match. Following exposure of HOD mice to HOD RBCs, anti-HOD IgM and IgG was assessed on Days 5 and 14 post-transfusion, respectively, by flow-crossmatch. For (B), ****=p<.0001, ***=p<.0002 and *=p<.05 by One-way ANOVA with Tukey's multiple comparisons test. Means ± s.d. shown.

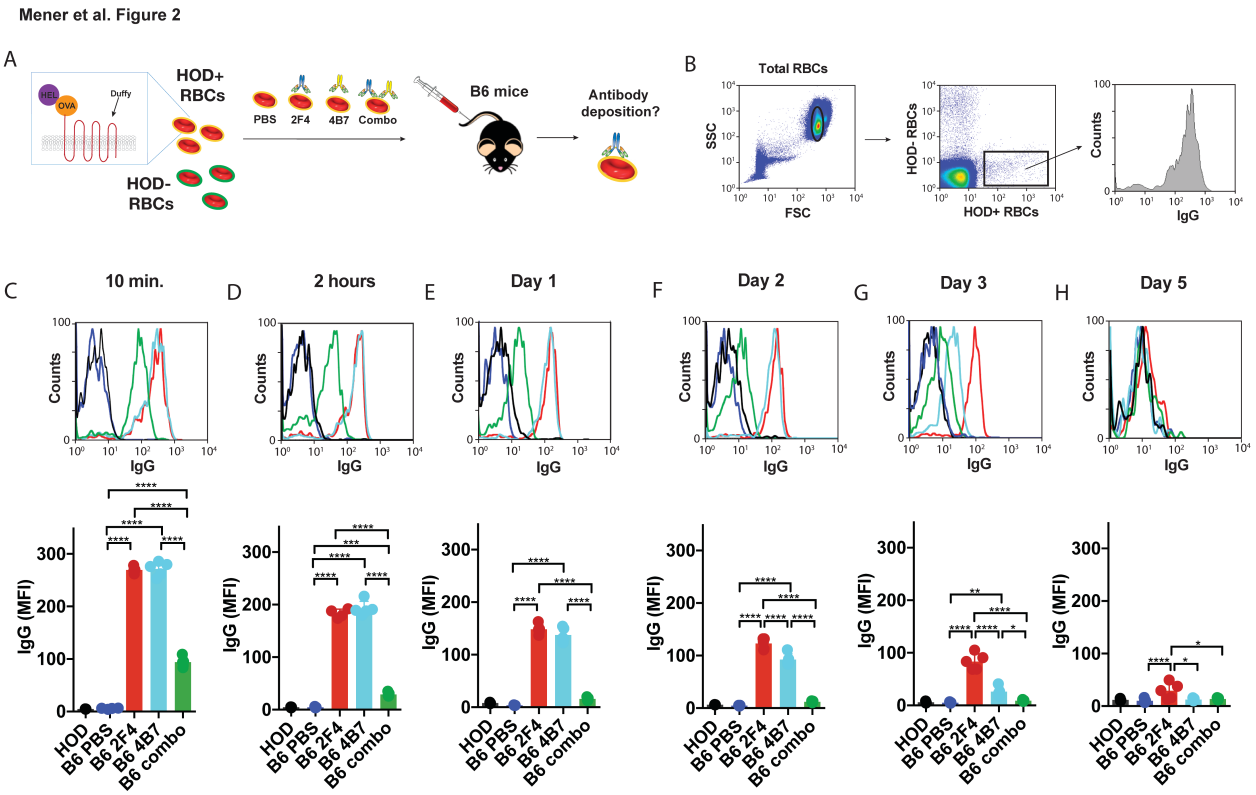


Figure 2: Anti-HEL monoclonal antibodies are detected on HOD RBCs following transfusion. (A) B6 mice were exposed to DiI-labeled HOD$^+$ RBCs and control DiO-labeled HOD$^-$ RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 (combo)) or absence (PBS) of anti-HEL monoclonal antibodies. (B) The deposition of surface IgG antibody specifically on HOD$^+$ RBCs was probed at 10 minutes (C), 2 hours (D), Day 1 (E), Day 2 (F), Day 3 (G) and Day 5 (H). For (C-H), ****=p<.0001, ***=p<.0008, **=p< .008 and *=p<.05 by One-way ANOVA with Tukey's multiple comparisons test. Means ± s.d. shown.

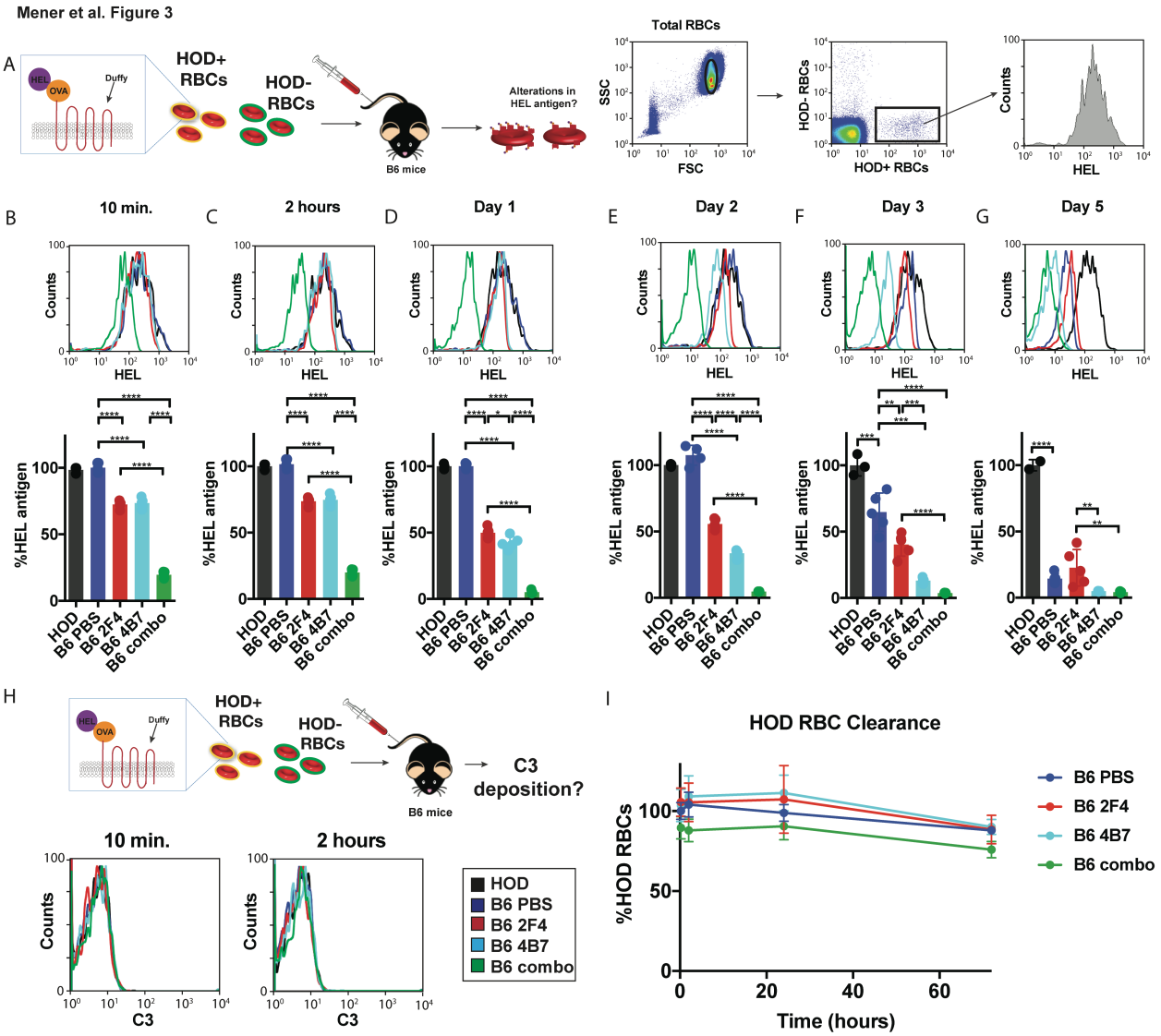


Figure 3: Anti-HEL antibodies induce a decrease in the level of detectable HEL antigen and fail to deposit complement or induce clearance. (A) B6 mice were exposed to a mixture of DiI-labeled HOD$^+$ RBCs and control DiO-labeled HOD$^-$ RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 (combo)) or absence (PBS) of anti-HEL monoclonal antibodies. (B-G) Post-transfusion, the level of HEL antigen on circulating HOD$^+$ RBCs was assessed at 10 minutes (B), 2 hours (C), Day 1 (D), Day 2 (E), Day 3 (F) and Day 5 (G). Level of HEL antigen was measured by percentage of HEL antigen normalized to the HOD control group. (H) HOD$^+$ RBCs in the absence (PBS) or

presence of anti-HEL monoclonal antibodies (2F4 and 4B7) individually or in combination (combo) were examined for C3 deposition following exposure in B6 mice at 10 minutes and 2 hours post-transfusion. (I) Clearance of HOD^{+} RBCs in B6 mice exposed to DiI-labeled HOD^{+} RBCs and control DiO-labeled HOD^{-} RBCs in the presence or absence of anti-HEL monoclonal antibodies (2F4 and 4B7) individually or in combination (combo). For (B-G), ****=p<.0001, ***=p<.0006, **=p<.002 and *=p<.05 by One-way ANOVA with Tukey's multiple comparisons test. For (I), statistical analysis was performed using Two-way ANOVA with Dunnett's multiple comparisons test. Means ± s.d. shown.

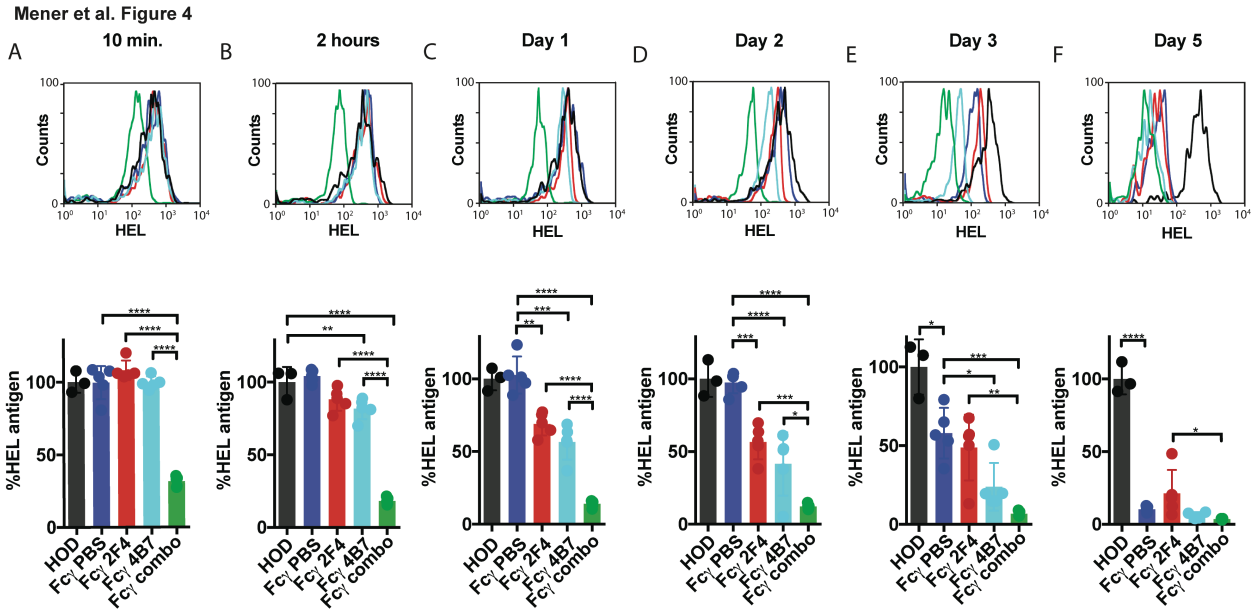


Figure 4: Anti-HEL antibodies induce a decrease in the level of detectable HEL antigen in FcγR KO mice. (A-F) FcγR KO mice (Fcγ) were exposed to a mixture of DiI-labeled HOD^+ RBCs and control DiO-labeled HOD^- RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 (combo)) or absence (PBS) of anti-HEL monoclonal antibodies. RBCs were collected and the percentage of detectable HEL antigen, normalized to control HOD mice exposed to HOD^+ RBCs, was measured at 10 minutes (A), 2 hours (B), Day 1 (C), Day 2 (D), Day 3 (E) and Day 5 (F). For (A-F), ****=p<.0001, ***=p<.003, **=p<.009 and *=p<.05 by One-way ANOVA with Tukey's multiple comparisons test. Means ± s.d. shown.

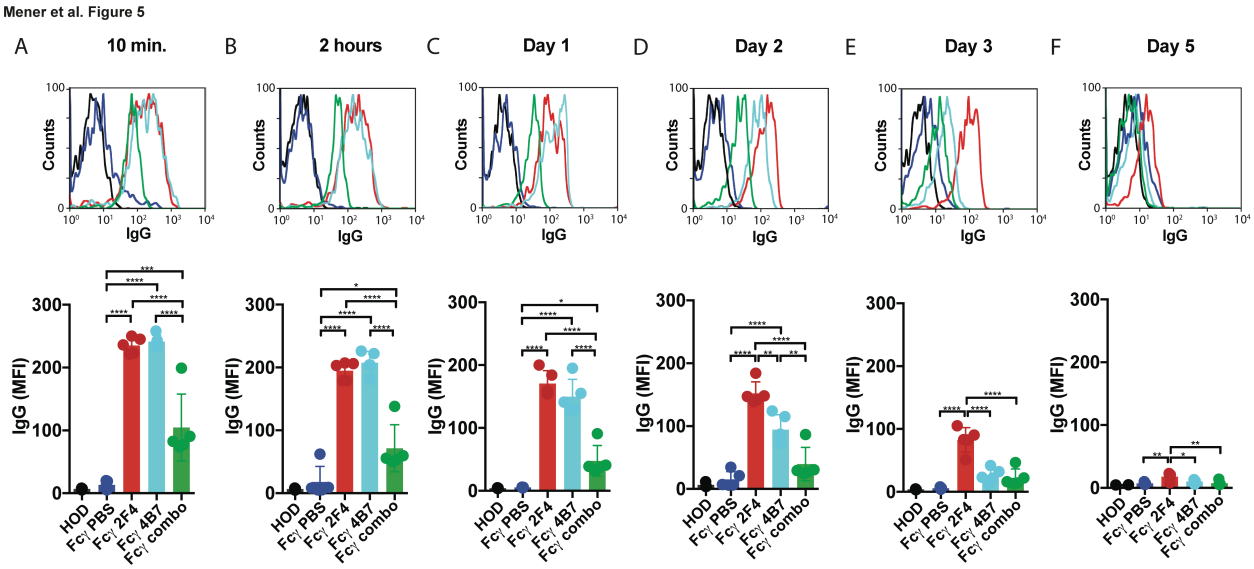


Figure 5: HOD RBCs exposed to anti-HEL antibodies display decreased levels of bound antibody in FcγR KO mice. (A-F) FcγRKO mice (Fcγ) were exposed to a mixture of DiI-labeled HOD^+ RBCs and control DiO-labeled HOD^- RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 (combo)) or absence (PBS) of anti-HEL monoclonal antibodies. Post-transfusion, RBCs were assessed for IgG deposition via the direct antiglobulin test at 10 minutes (A), 2 hours (B), Day 1 (C), Day 2 (D), Day 3 (E) and Day 5 (F). For (A-F), ****=p<.0001, ***=p<.0006, **=p<.002 and *=p<.05 by One-way ANOVA with Tukey's multiple comparisons test. Means ± s.d. shown.

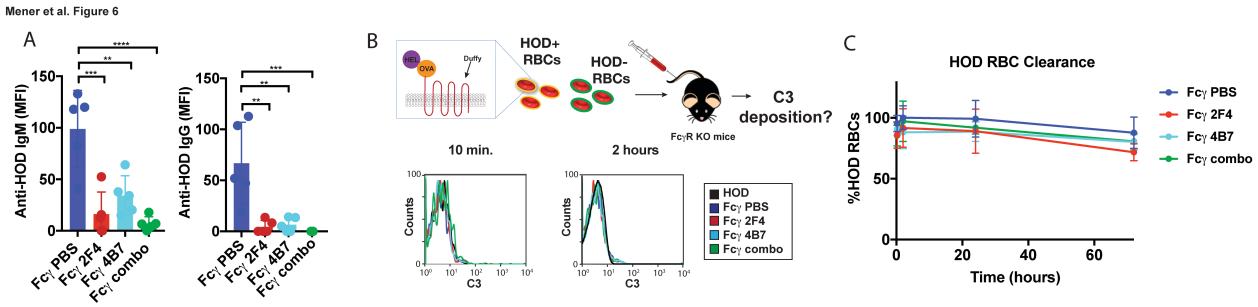


Figure 6: HOD RBCs exposed to anti-HEL antibodies in FcγR KO mice results in dampening of the anti-HOD antibody response without complement deposition nor clearance of HOD RBCs. (A) Serum was assessed for the presence of anti-HOD IgM on Day 5 and anti-HOD IgG on Day 14 post-transfusion of HOD^+ RBCs in FcγR KO mice (Fcγ) by flow cross-match. (B) HOD RBCs in the absence (PBS) or presence of anti-HEL monoclonal antibodies (2F4 and 4B7) individually or in combination (combo) were examined for C3 deposition following exposure in FcγR KO mice (Fcγ) at 10 minutes and 2 hours post-transfusion. (C) Clearance of HOD RBCs in FcγR KO mice (Fcγ) exposed to DiI-labeled HOD^+ RBCs and control DiO-labeled HOD^- RBCs in the presence or absence of anti-HEL monoclonal antibodies (2F4 and 4B7) individually or in combination (combo). For (A), ****=p<.0001, ***=p<.0003 and **=p<.003 by One-way ANOVA with Tukey's multiple comparisons test. For (C), statistical analysis was performed using Two-way ANOVA with Dunnett's multiple comparisons test. Means ± s.d. shown.

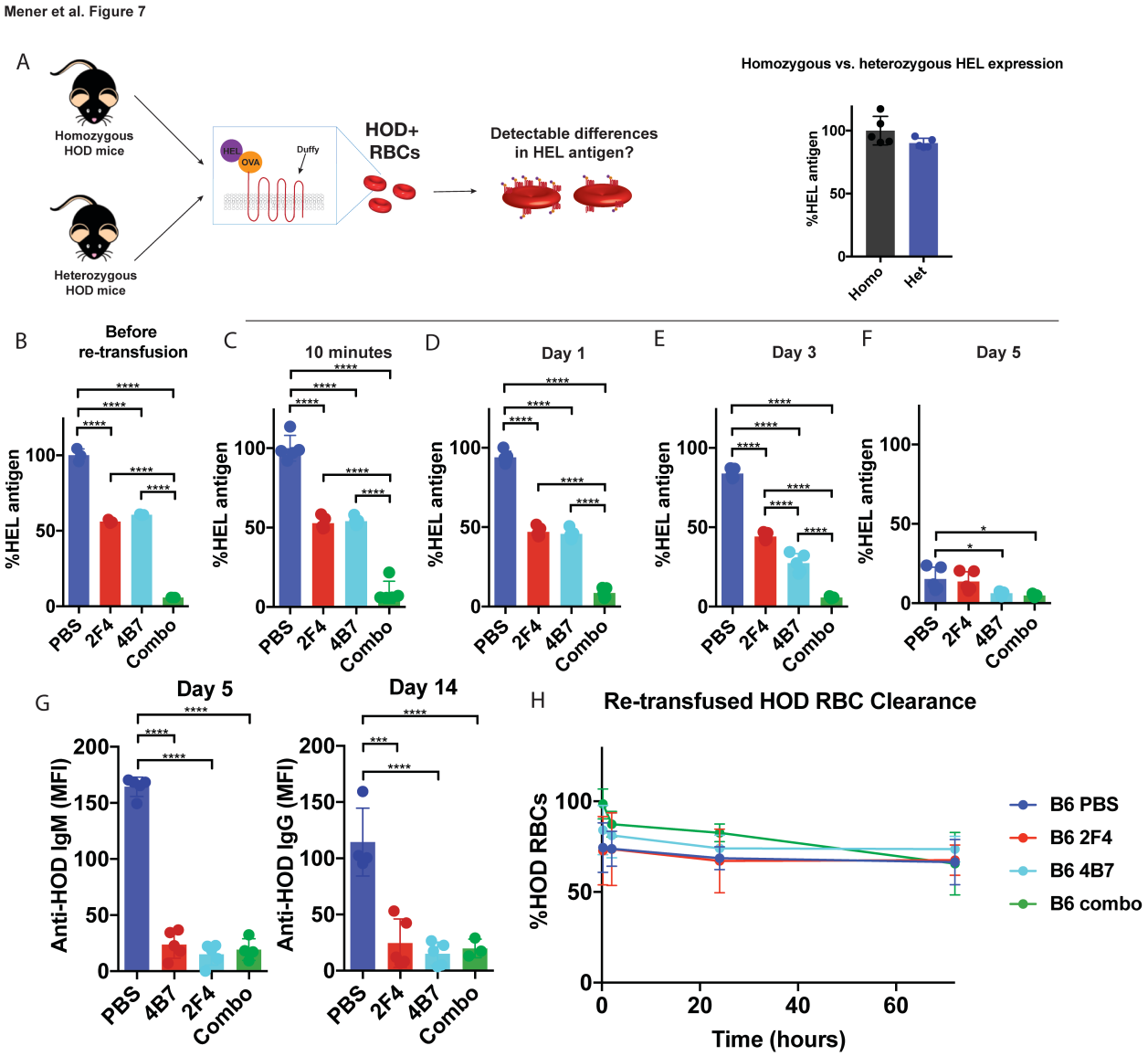


Figure 7: Re-transfusion of HOD RBCs with varying levels of HEL antigen correlates with dampening of the anti-HOD antibody response without clearance of HOD RBCs. (A) HEL antigen on homozygous and heterozygous HOD mice was assessed by flow cytometry. (B) B6 mice were exposed to HOD^{+} RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 (combo)) or absence (PBS) of anti-HEL monoclonal antibodies. The level of detectable HEL antigen was then assessed followed by re-transfusion of RBCs into naïve B6 recipients. (C-F) Following re-transfusion, the level of HEL antigen was detected at 10 minutes (C), Day 1 (D), Day 3 (E) and Day 5

(F). (G) Serum was collected from re-transfused recipients and the development of anti-HOD IgM and IgG was assessed on Day 5 and 14 post-transfusion, respectively, by flow cross-match. (H) Clearance of anti-HEL monoclonal antibody-antigen modulated HOD^{+} RBCs following re-transfusion into naïve B6 mice. For (B-G), ****=p<.0001, ***=p<.0002 and *=p<.05 significant by One-way ANOVA with Tukey's multiple comparisons test. For (H), statistical analysis was performed using Two-way ANOVA with Dunnett's multiple comparisons test. Means ± s.d. shown.

Mener et al. Figure 8

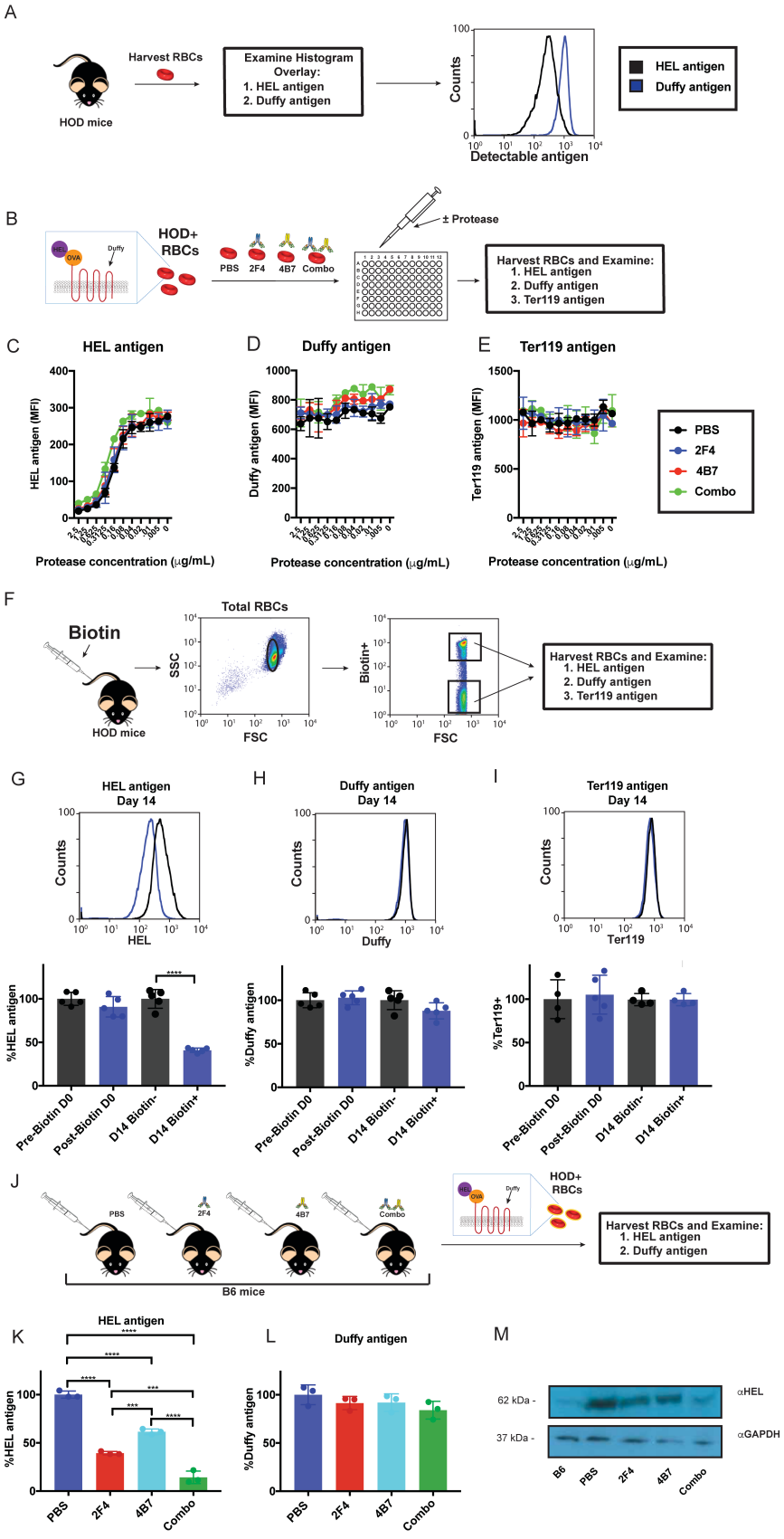
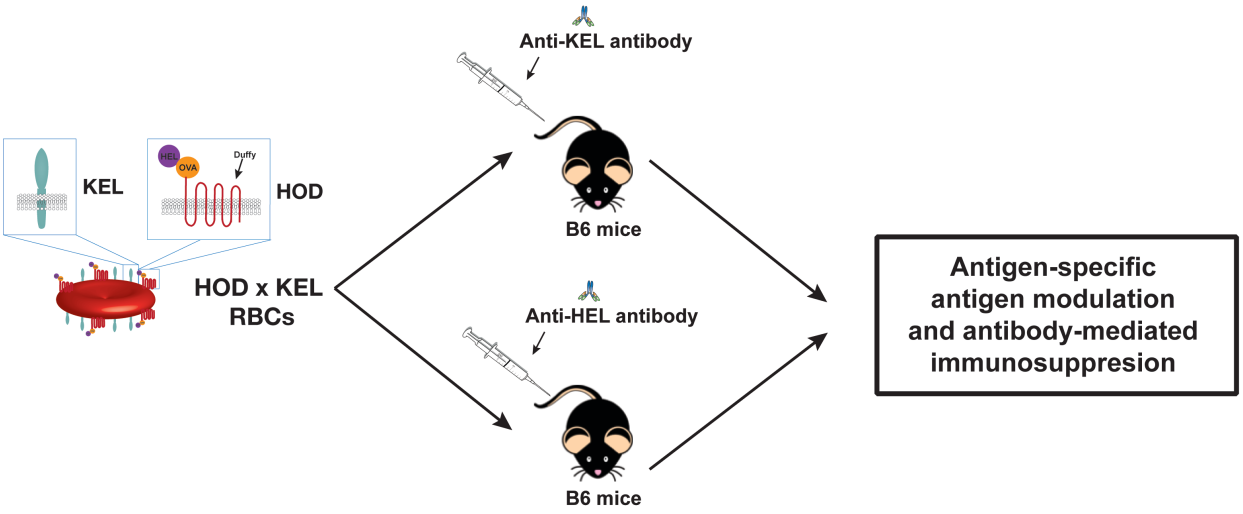


Figure 8: HEL antigen decreases over time on HOD RBCs as they age but not with protease treatment *in vitro*. (A) HOD^+ RBCs stained for the level of detectable HEL and Duffy antigens, followed by overlay of histograms. (B) HOD^+ RBCs were treated with proteases in the presence or absence of anti-HEL monoclonal antibodies. Following treatment, the level of detectable HEL (C), Duffy (D) and Ter119 (E) antigens were measured by flow cytometry. (F) HOD mice were biotinylated via intravenous injection of NHS-sulfo-biotin, followed by tracking biotin positive RBCs *in vivo* over time. (G-I) RBCs were harvested and evaluated for levels of HEL (G), Duffy (H) and Ter119 (I) by flow cytometry on day 0 (D0) or day 14 (D14) post-biotinylation. (J) B6 mice were either not immunized (PBS) or passively immunized with anti-HEL monoclonal antibodies (2F4, 4B7 or a combination of 2F4 and 4B7 (combo)), followed by exposure to HOD^+ RBCs. 2 days following transfusion, HOD^+ RBCs were harvested. The level of detectable HEL antigen (K) and Duffy antigen (L) were then measured by flow cytometry. RBCs were also subjected to western blot analysis for HEL (M). For (C-E), statistical analysis was performed using Two-way ANOVA with Tukey's multiple comparison's test. For (G-L), ****=$p<.0001$ and ***=$p<.0003$ by One-way ANOVA with Tukey's multiple comparisons test. Means ± s.d. shown.

Chapter 3: Antibody-Mediated Immunosuppression by Antigen Modulation is Antigen-Specific

Visual Abstract



Abstract

Alloantibodies developing after exposure to red blood cell (RBC) alloantigens can complicate pregnancy and transfusion therapy. The only method currently available to actively inhibit RBC alloantibody formation is administration of antigen-specific antibodies, a phenomenon termed antibody-mediated immune suppression (AMIS). A well-known example of AMIS is RhD immune globulin (RhIg) prophylaxis to prevent anti-D formation in RhD-negative individuals. However, whether AMIS is specific or may additionally impact alloimmunization to other antigens on the same RBC remains unclear. To evaluate the specificity of AMIS, we passively immunized antigen-negative recipients with anti-KEL or anti-hen egg lysozyme (HEL) antibodies, followed by transfusion of murine RBCs expressing both the HOD (HEL-ovalbumin-Duffy) and human KEL antigens (HOD x KEL RBCs). Significant IgG deposition on transfused HOD x KEL RBCs occurred in all passively immunized recipients. However, complement was detected on HOD x KEL RBCs only in the presence of anti-KEL antibodies, recapitulating previous findings using single antigen-expressing RBCs. Moreover, antigen modulation of the KEL antigen occurred on transfused RBCs only in anti-KEL treated recipients, while HEL antigen levels decreased only in the presence of anti-HEL antibodies. Western blot analysis demonstrated that antigen modulation represented antigen removal and was specific to the passively immunized antibody. In addition, antigen-specific modulation correlated with antigen-specific AMIS, with anti-KEL treated recipients forming antibodies to the HOD antigen and anti-HEL treated recipients developing antibodies to the KEL antigen. Together, these results demonstrate

that passively administered antibodies can selectively inhibit the immune response to a specific antigen.

Introduction

Antibodies to red blood cell (RBC) antigens can develop after exposure to RBC alloantigens during pregnancy or transfusion therapy. Such antibodies can complicate transfusion therapy by causing hemolytic transfusion reactions (HTR), or pregnancies by causing hemolytic disease of the fetus and newborn (HDFN) (1-3). Additionally, alloantibodies to RBC antigens can decrease the therapeutic efficacy of transfused RBCs and hinder finding compatible RBCs for future transfusions (4). Excluding antigen matching protocols to reduce alloimmunization risk (5, 6), the sole therapeutic intervention currently available to prevent RBC alloantibody development is polyclonal RhD-immune globulin (RhIg) (7). Prophylactic administration of RhIg to RhD-negative women successfully prevents the generation of *de novo* anti-RhD antibodies associated with pregnancy through a process termed antibody-mediated immune suppression (AMIS) (8-12), whereby passively acquired antibody inhibits sensitization to a given antigen. While AMIS is a successful clinical intervention, no such therapeutic options exist to prevent formation of other clinically significant non-RhD alloantibodies. This likely reflects both our inability to fully understand RhIg-related AMIS and variability in the mechanism and outcome of AMIS depending on the specific antigens or antibodies involved (13-19).

Several mechanisms by which AMIS exerts its inhibitory effect have been postulated and tested, aided recently by the development of improved animal models. These include rapid induction of RBC clearance, antigen masking with steric hindrance of B cell receptors, inhibition of B cell responses through inhibitory Fcγ receptor (FcγR) engagement, and direct antigen modulation, which refers to removal of antigen from the

cell surface and renders a cell antigen-negative as it persists in circulation (15, 16, 20, 21). RBC clearance has been regarded as the primary mechanism of AMIS and occurs following passive immunization to some antigens, including RhD (22). However, recent evidence implicates antigen modulation as another mechanism by which AMIS can occur independent of RBC clearance (13, 14).

While previous reports support antigen modulation as a mechanism of AMIS, these studies were performed using murine models where RBCs express only one foreign antigen (13, 14, 19, 23, 24). Therefore, it remains unclear whether antigen modulation is a potential mechanism of AMIS in the context of multiple RBC antigens expressed simultaneously, which is more clinically-relevant, and whether this type of AMIS is antigen-specific. Previous observations suggest AMIS resulting from RBC clearance is non-specific and inhibits sensitization to additional RBC alloantigens. For example, before RhIg availability, decreased rates of anti-RhD alloimmunization were recognized in RhD-negative mothers who were also ABO-incompatible with their babies (25-27). Another study in human volunteers found suppression of RhD sensitization in RhD-negative KEL-negative men exposed to RhD-positive KEL-positive RBCs and passively immunized with anti-KEL IgG (25). These observations contribute to the notion that AMIS is not antigen-specific and instead reflects rapid antibody-induced clearance of transfused cells before the adaptive immune response can detect foreign RBC antigens. More recent findings reported on the lower rates of human leukocyte antigen sensitization in previously pregnant RhD-negative women, who presumably received RhIg, compared to previously pregnant RhD-positive women, again suggesting that AMIS may not be antigen-specific (28). Nevertheless, some women treated with RhIg do become

alloimmunized to non-RhD fetal RBC antigens, highlighting conflicting evidence regarding whether AMIS can be antigen-specific and whether specificity depends on the antigens involved.

Since the antigen-specific nature of AMIS has not been evaluated in the context of antigen modulation, we sought to clarify whether antigen modulation plays a role in AMIS when multiple foreign antigens are present on the RBC surface and whether this mechanism of AMIS is antigen-specific. In particular, we investigated whether antibody-specificity to one RBC antigen induces antigen-specific antigen modulation that results in antigen-specific AMIS. To address this, we generated transgenic mice expressing both the model hen egg lysozyme (HEL)-ovalbumin (OVA)-human Duffy (HOD) antigen and the human KEL antigen on RBCs (HOD x KEL RBCs). We then transfused wild type recipients, passively immunized with anti-KEL or anti-HEL antibodies, or PBS control, with HOD x KEL RBCs. We assessed changes in the level of detectable antigen, as well as any impact of antigen-specific antibodies on the immune response to another antigen present on transfused RBCs. Here we report that antigen-specific antibodies cause antigen-specific antigen modulation and result in antigen-specific AMIS on RBCs bearing multiple foreign antigens.

Materials and Methods

Mice

All mice were housed in the Emory Division of Animal Resources and Husbandry facilities and treated in accordance with protocols approved by the Emory University Institutional Animal Care and Use Committee. HOD x KEL transgenic mice were generated as described previously (29, 30). Wild type C57BL/6 (WT) recipient mice were purchased from Charles River (Montreal, QC, Canada). Male and female mice were used between 8 to 12 weeks of age.

Passive Immunization & Transfusion

WT recipients were passively immunized with either anti-KEL polyclonal antibody, generated as previously described (14), or a combination of two anti-HEL monoclonal antibodies (clones 2F4 and 4B7, both from Bio X Cell, West Lebanon, NH) previously shown to induce AMIS to the HEL antigen (31), or received PBS control by tail vein injection prior to transfusion. HOD x KEL or WT RBCs were collected into acid citrate dextrose (ACD, BD, Franklin Lakes, NJ) and washed 3x in PBS. HOD x KEL^+ and HOD x KEL^- RBCs were labeled with DiI and DiO as previously described (19, 32). Each recipient was transfused a total volume of 300 μl, including 50 μl packed HOD x KEL RBCs and 50 □l packed WT RBCs, the adjusted equivalent of one human RBC unit.

Staining and Antibody Identification by Flow Cytometry

Platelets and splenocytes were collected from WT and HOD x KEL mice as previously described (33). Following isolation, platelets and splenocytes were stained with lineage-

specific markers, CD41 and CD45, respectively (BD, Franklin Lakes, New Jersey) as previously described (33). RBCs were collected from recipients either prior to transfusion for characterization of HOD and KEL expression or following transfusion, at 1 hour, 2 hours and 24 hours post-transfusion and stained for flow cytometry as previously described (23, 34-36). Briefly, RBCs were stained with anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) for assessment of bound antibody, with biotin anti-mouse complement component 3 (C3; Cedarlane, Burlington, NC) for assessment of total complement, or biotin anti-mouse C3b (Cedarlane, Burlington, NC) for detection of active complement, followed by streptavidin (BD, Franklin Lakes, NJ). KEL antigen was measured using anti-KEL polyclonal antibody followed by anti-mouse IgG secondary (Jackson Immunoresearch, West Grove, PA). HEL antigen was measured using anti-HEL monoclonal antibodies followed by secondary anti-mouse IgG. Ter119 was measured using Ter119 APC (BD, Franklin Lakes, NJ). To assess for the development of *de novo* anti-KEL and anti-HOD IgM and IgG antibodies, serum was flow cross-matched as previously described (14, 24, 33). All samples were run on a BD FACSCalibur flow cytometer and analyzed using FlowJo software (Tree Star Inc. Ashland, OR).

Immunofluorescent super-resolution microscopy

1 mL of a 1:10 dilution of poly-L-lysine in PBS (Sigma 0.01% stock solution) was added to each 15 mm diameter glass coverslip. Coverslips were washed 3x with PBS then air-dried. Anti-HEL antibody was directly labeled using Alexa Fluor™ 488 NHS Ester (Succinimidyl Ester, ThermoFisher Scientific, A20000). HOD x KEL RBCs were stained with polyclonal anti-KEL antibody, followed by anti-mouse IgG-Alexa 555 (Invitrogen,

Carlsbad, CA). This was followed by staining with directly labeled anti-HEL antibody. Stained HOD x KEL RBCs were added to coverslips and incubated for 30 minutes on ice, followed by fixation with 1 mL of 1% paraformaldehyde in PBS overnight. Imaging was performed with a Nikon N-SIM super resolution microscope.

Western Blot Analysis

Isolated RBCs were lysed in 5 mmol/L sodium phosphate (pH 7.5) with protease inhibitor cocktail (Sigma Roche, St. Louis, MO). RBC ghosts were collected by centrifugation and washed with lysis buffer until translucent, as previously described (14, 37). Western blot antibodies included anti-KEL antibody (Abcam, clone MM0435-12X3, diluted 1:250 in blocking buffer), rabbit polyclonal anti-HEL antibody (Abcam, ab391, 1:5,000), and goat anti-rabbit HRP (Abcam, ab205718, 1:10,000) for detection. Mouse monoclonal anti-GAPDH (clone: GA1R, ThermoFisher Scientific, Waltham, MA, 1:1,000), followed by goat anti-mouse IgG1 HRP (Bethyl Laboratories, Montgomery, TX, 1:10,000) was applied after stripping membranes with 25 mM glycine-HCl with 1% SDS, pH 2. Membranes were developed using HyGlo ECL (Denville Scientific Inc. Holliston, MA) and protein densitometry performed using ImageJ (Bethesda, MD).

Statistics

Statistical significance was determined using GraphPad Prism (GraphPad Software, San Diego, CA) and defined as $p<0.05$. Unpaired student's t-test was used to compare two groups and One-way ANOVA with Tukey's multiple comparisons test was utilized to compare more than two groups.

Results

As previous studies suggested that AMIS to human RBC antigens might be non-specific (25, 28), we sought to determine whether AMIS could demonstrate antigen-specific restriction. To accomplish this, we generated mice whose RBCs express two distinct antigens, HOD and KEL, as previously reported (Figure 1A) (38). To confirm expression of HOD and KEL on RBCs, RBC ghosts were generated from WT or HOD x KEL RBCs and subjected to western blot analysis, which showed expression of the HOD and KEL RBC antigens only on HOD x KEL RBCs (Figure 1B). The expression of HOD and KEL individually, as well as dual staining of HOD and KEL together, was further shown to be restricted to RBCs only (Figure 1C), with no expression on other cell populations, such as platelets (Figure 1D) or white blood cells (Figure 1E). As antibody binding and saturation levels may differ *in vitro* versus *in vivo,* we next determined whether saturation of HOD x KEL RBCs occurred *in vitro* and whether this saturation reflected antibody binding *in vivo*. To accomplish this, we incubated HOD x KEL RBCs with anti-KEL or anti-HEL antibody *in vitro*. We also exposed WT mice to HOD x KEL RBCs following passive immunization with anti-KEL or anti-HEL antibodies and assessed antibody binding to transfused RBCs by flow crossmatch (Figure 2A). Saturation of HOD x KEL RBCs by anti-KEL occurred over a wide range of anti-KEL dilutions (Figure 2B). Importantly, similar saturation of HOD x KEL RBCs was observed *in vivo* as compared to the *in vitro* binding of antibodies to HOD x KEL RBCs from the serum of anti-KEL immunized mice to a saturating concentration of anti-KEL antibody (Figure 2C). We also observed saturation of HOD x KEL RBCs by anti-HEL antibodies *in vitro* over a wide range of concentrations (Figure 2D), with saturation of HOD x KEL

RBCs likewise occurring *in vivo*, when we compared the binding of antibodies from the serum of anti-HEL passively immunized mice to a saturating concentration of anti-HEL antibody *in vitro* (Figure 2E). These results suggest that saturation of HOD x KEL RBCs can occur both *in vitro* and *in vivo* utilizing either anti-KEL or anti-HEL antibodies.

Next, as antibody directed against one RBC antigen may sterically hinder the binding of antibody against a different RBC antigen when expressed on the same RBC, we tested whether incubation of HOD x KEL RBCs with anti-KEL antibody prior to anti-HEL antibody affected the ability of anti-HEL antibodies to detect the HEL antigen (Figure 2F). HEL antigen was detected by anti-HEL antibodies on HOD x KEL RBCs over a wide range of anti-KEL dilutions, suggesting that anti-KEL binding does not sterically hinder the ability of anti-HEL antibody to bind HOD x KEL RBCs (Figure 2G). The detectability of the HOD and KEL RBC antigens was further confirmed by super resolution microscopy, which demonstrated anti-HEL and anti-KEL binding on the same RBCs, but with distinct localization (Figure 2H). Together, these results suggest that although HOD and KEL are expressed on the same RBCs, antibody binding to one RBC antigen does not appear to influence antibody binding to the other RBC antigen.

Antibody and complement deposition on transfused HOD x KEL RBCs

To examine the specificity in the development of the immune response to either RBC antigen when they are expressed on the same RBC, we first tracked HOD x KEL RBCs post-transfusion by labeling with the lipophilic dye DiI and mixing with DiO-labeled tracer WT RBCs. WT mice, passively immunized with anti-KEL or anti-HEL antibodies or injected with PBS, were transfused with a 1:1 mixture of HOD x KEL and

WT RBCs (Figure 3A). Antibody deposition on transfused HOD x KEL cells was determined using a direct antiglobulin test (DAT) by flow cytometry at 1 hour, 2 hours, and 24 hours post-transfusion. Recipients that received anti-KEL or anti-HEL antibodies showed bound antibody on the surface of transfused HOD x KEL RBCs, unlike the PBS control group, which demonstrated no antibody binding (Figure 3B). Antibody deposition decreased over time in groups receiving either the anti-KEL or anti-HEL antibodies (Figure 3B). Together, these data reflect the ability of these antibodies to selectively bind to their target antigen on transfused HOD x KEL RBCs *in vivo*.

As antibodies bound to the surface of RBCs have been shown to fix complement, we next investigated whether complement was deposited on transfused HOD x KEL RBCs. Complement deposition was determined by measuring total (C3) and active (C3b/iC3b) complement on transfused RBCs at 1 hour, 2 hours and 24 hours post-transfusion by flow cytometry. Complement deposition on transfused HOD x KEL RBCs was significant only in recipient mice passively immunized with anti-KEL antibodies (Figure 3). Specifically, total complement deposited on transfused cells in the anti-KEL treated mice and increased over time (Figure 3C), peaking at 24 hours post-transfusion, while C3b/iC3b complement peaked at 1 hour (Figure 3D), indirectly indicating that complement on HOD x KEL RBCs at 24 hours post-transfusion was residual bound complement products. These findings are consistent with previous reports using RBCs expressing either the KEL or HOD antigens individually, where transfusion of KEL RBCs in the presence of anti-KEL antibodies causes complement deposition but transfusion of HOD RBCs in the presence of anti-HEL antibodies does not (36, 39). Thus, even when HOD and KEL are expressed on the same RBC, each antigen retains its

individual property regarding complement deposition as when either HOD or KEL are expressed separately.

HOD x KEL RBC clearance in passively immunized recipients

Deposition of antibodies and complement on the RBC surface promotes cell clearance from the circulation by opsonizing cells for removal (40-42). Since transfused HOD x KEL RBCs were bound by antibody and, in the case of anti-KEL immunized mice, complement as well, we assessed RBC clearance in all recipient groups. To do so, we directly compared the ratio of HOD x KEL RBCs (HOD x KEL^+) to the WT tracer population (HOD x KEL^-) at 1 hour, 2 hours, and 24 hours post-transfusion (Figure 3E). HOD x KEL RBCs were not cleared in mice receiving anti-HEL antibody or PBS (Figure 3E). In contrast, in mice that received anti-KEL antibody, approximately 20% of transfused HOD x KEL RBCs cleared (36, 39). Importantly, RBC survival plateaued in this group and did not further significantly decrease between 4 hours and 24 hours post-transfusion (Figure 3E), which coincided kinetically with decreasing bound antibody (Figure 3B). These results suggest that non-cleared HOD x KEL RBCs persist by modification of antibody targets, such as antigen modulation, which has been shown to occur with single positive RBCs (24). These results further suggest that the unique properties of each antigen are retained even when expressed on the same RBC, thus recapitulating findings when either antigen is expressed on RBCs individually.

Passive immunization results in antigen-specific antigen modulation

Antigen modulation has been shown to account for AMIS in models where

transfused RBCs express a single foreign antigen and are not fully cleared from the circulation, likely related to decreased antibody binding on consequently antigen-negative cells (13, 14). Since the majority of transfused HOD x KEL RBCs survived in our model, despite antibody binding, we next assessed whether transfused HOD x KEL RBCs underwent antigen loss. The levels of detectable KEL, HEL, and Ter119 antigens were quantified on HOD x KEL RBCs after transfusion into passively immunized or PBS control-treated recipients by flow cytometry. Whereas KEL antigen levels decreased on transfused HOD x KEL RBCs over time in anti-KEL treated mice, levels were unaffected in mice given anti-HEL antibodies or PBS (Figure 4A), indicating that passive immunization with anti-KEL antibody causes only KEL antigen loss, despite HOD and KEL antigens on the same RBCs. Similarly, HEL antigen levels significantly decreased in mice that had received anti-HEL antibodies, but not in mice that had received anti-KEL antibodies or PBS control (Figure 4B), again indicating that antigen modulation on the RBC surface is antigen-specific. The decreases in KEL or HEL antigen levels on transfused HOD x KEL RBCs in passively immunized recipients was not due to non-specific membrane alterations of RBCs, as there was no difference among the groups in the levels of Ter119, a protein expressed on erythroid cells (Figure 4C).

To ensure that antibody-induced decreases in the level of detectable antigen truly reflected antigen-specific removal, we next quantified the individual antigens on transfused RBCs by western blot analysis. WT mice were passively immunized with PBS, anti-KEL or anti-HEL antibody and subsequently exposed to HOD x KEL RBCs. Post-transfusion, the levels of KEL and HEL antigen were tracked over time until significant decreases occurred in the corresponding passively immunized mice (Figure

5A-B), with no antigen modulation observed for the Ter119 antigen (Figure 5C). Once antigen modulation was detected by flow cytometry, RBCs were harvested to prepare RBC ghosts. Western blot analysis showed that anti-KEL antibody induced antigen-specific removal of the KEL antigen, whereas anti-HEL antibody induced antigen-specific removal of the HEL antigen, consistent with the flow cytometry data (Figure 5D-F). These results demonstrate that passive immunization results in antigen loss of KEL or HEL antigens, but only for the corresponding antigen and without any detectable effect on other antigens.

Suppression of antibody development (AMIS) is antigen-specific

Previous studies demonstrated a correlation between antigen modulation and suppression of sensitization in AMIS models where RBCs express a single foreign antigen.(13, 14) Therefore, we next tested recipient serum for newly generated anti-KEL and anti-HOD antibodies to determine whether antigen modulation correlated with AMIS and whether suppression was antigen-specific (Figure 6A). Anti-KEL IgM was detected on days 5 and 7 post-transfusion in mice that received PBS control or passive anti-HEL immunization (Figure 6B). However, anti-KEL IgM was not detected at any time-points in mice that received anti-KEL immunization. Likewise, PBS control and anti-HEL treated recipients developed anti-KEL IgG on days 7 and 14, but anti-KEL treated recipients did not (Figure 6C). Thus, passive immunization with anti-KEL protected recipients from developing endogenous anti-KEL antibodies following exposure to HOD x KEL RBCs, while passive immunization with anti-HEL had no effect and resulted in an anti-KEL response similar to unimmunized PBS control-treated mice.

Determination of an anti-HOD response was similarly performed and yielded results consistent with the anti-KEL data. On days 5 and 7 post-transfusion, significant amounts of anti-HOD IgM was detected in both PBS control-treated and anti-KEL treated mice, but not in mice that received passive anti-HEL immunization (Figure 6D). Additionally, anti-HOD IgG was detected at all time-points in PBS control-treated and anti-KEL-treated mice, but not in mice that had received anti-HEL immunization (Figure 6E). Interestingly, anti-KEL immunized mice had a slightly, but significantly and reproducibly, increased anti-HOD IgG response compared to unimmunized PBS-control treated mice (Figure 6E), perhaps related to increased RBC clearance in anti-KEL treated recipients (Figure 3E). Taken together, we find that mice passively immunized to KEL do not develop anti-KEL antibodies, but do develop anti-HOD antibodies, after exposure to RBCs bearing both antigens, indicating the specificity of the AMIS effect. Conversely, mice passively immunized to HEL do not develop anti-HOD antibodies, but do develop anti-KEL antibodies similarly to unimmunized mice, again indicating that AMIS is antigen-specific. Thus, anti-KEL or anti-HEL immunization results in antigen-specific AMIS that correlates with antigen modulation.

Discussion

Here we report that passive immunization results in antigen-specific antigen modulation and suppression of antibody development. Using a novel AMIS model, in which mice were exposed to RBCs bearing two foreign antigens and immunized with antibodies to either antigen individually, we tested the specificity of AMIS and any consequence on other non-specific antigens. Murine RBCs expressing both KEL and HOD RBC antigens demonstrated similar clearance kinetics, antigen levels, and antibody development after transfusion into passively immunized mice as is observed with KEL-only or HOD-only RBCs. Furthermore, our data showed that antigen-specific immunization does not impact antibody binding, complement deposition, or antigen modulation to other RBC antigens.

Differences between the various animal models and clinical observations in humans have complicated our understanding of AMIS and how it occurs, but likely highlight the variability and multi-mechanistic nature of AMIS depending on the particular antigens and antibodies involved. We intentionally used HOD x KEL RBCs for our studies, as antigen modulation, and not RBC clearance, which may cause non-specific AMIS, appears to be the dominant mechanism in HOD-only or KEL-only models (13, 14). Recently, in an AMIS model using HOD-only RBCs and anti-ovalbumin antibodies, which resulted in both antigen modulation and RBC clearance, others reported that AMIS more closely correlates with saturating amounts of bound antibody and antigen modulation than with RBC clearance (13). Indeed, our data indicate that AMIS correlates more closely with IgG binding and antigen modulation than with RBC clearance. Even in the case of RhD, where RhIg results in significant RhD^+ RBC

clearance, we have previously found that RhIg induces antigen modulation in RhD^+ patients received RhIg as adjunct therapy for immune thrombocytopenic purpura (43). Thus, while RBC clearance occurs following antibody engagement with some antigens, the exact contribution of clearance in mediating AMIS requires further investigation.

One notable but not completely surprising finding was the boosted anti-HOD IgG response in mice immunized with anti-KEL antibodies. A possible explanation for the enhanced response is that anti-KEL-related RBC clearance creates a more inflammatory microenvironment. Inflammation is known to contribute to RBC alloimmunization, as TRIF-deficient mice do not make anti-HOD antibodies after HOD-only RBC transfusion (2, 44, 45) and other models demonstrate enhanced antibody responses to RBC alloantigens after immune cell priming to a different RBC alloantigen in the presence of inflammation (38). In contrast, in this study no boost in anti-KEL IgG occurred in mice immunized with anti-HEL antibody, as compared to PBS control treated mice, perhaps because anti-HEL does not induce RBC clearance and associated inflammatory signals. Taken together, these findings indicate that the exact contribution of clearance and antigen modulation to the developing immune response and AMIS may vary depending on the combination of passively administered antibody and RBC antigen(s) involved.

Understanding AMIS will allow for the development or improved understanding of various efficacious antibodies with clinical utility. This includes production of monoclonal antibodies to prevent RBC alloimmunization to a range of RBC alloantigens, inhibiting sensitization to non-RBC antigens by monoclonal or polyclonal agents, and even understanding how currently available antibody-based therapeutics affect RBCs. For example, recently we found that antigen modulation occurs on human RBCs after

treatment with daratumumab, an anti-CD38 monoclonal antibody used in the treatment of multiple myeloma (37). Furthermore, intravenous immunoglobulin is known to cause antibody deposition on RBCs and, in some cases, hemolytic anemia (46-49). Such findings highlight the importance of considering off-target effects of additional antibody-based therapeutic interventions.

As with all experimental models, limitations of using mice to recapitulate the pathophysiology of human disease must be considered. We and others consider the use of HOD or KEL RBCs in AMIS studies to be more physiologically relevant, especially compared to models using transfusion of sheep RBCs into mice (13, 19). As an advantage, our study models AMIS using two antigens that contain clinically relevant human proteins; however, allogeneic RBCs transfused to human patients express a panoply of foreign antigens, each of which may elicit unique mechanisms of AMIS in the presence of antibody. Such mechanistic multiplicity likely contributes to whether AMIS is antigen-specific, again depending on the particular antibodies and antigens involved. Whether antigen modulation occurs as a common mechanism of AMIS to additional antigens, and in humans, requires additional investigation.

In conclusion, we observed antigen-specific AMIS secondary to antigen modulation for both the HOD and KEL antigens in our model, indicating that the immune response following antigen-specific immunization does not impact antibody binding, antigen modulation, or antibody development to other RBC antigens. Our study provides insight into the specificity of AMIS resulting from antigen modulation, which may enlighten the development of additional therapeutics based on this type of immune suppression.

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Figures:

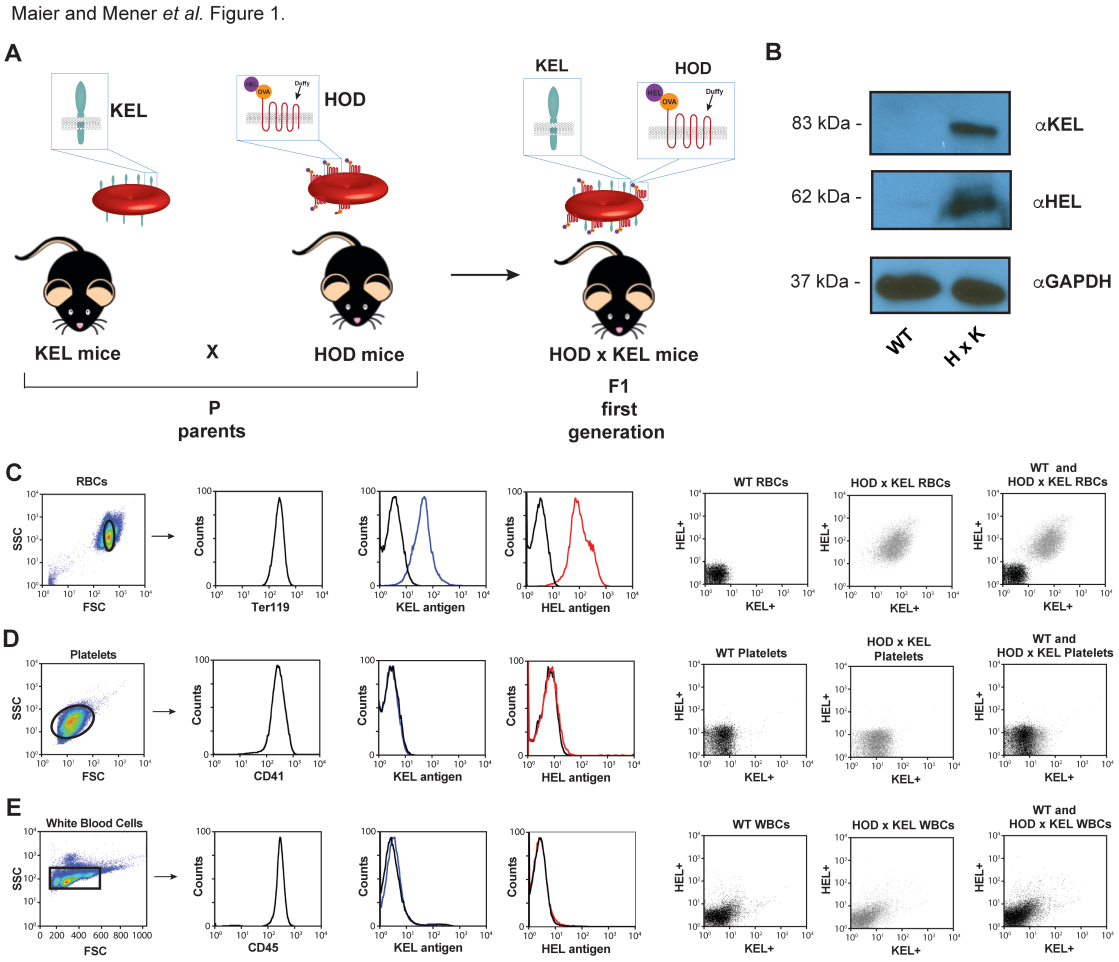

Figure 1: Characterization of HOD x KEL mouse model. (A) Mice that transgenically express HOD or KEL on their RBCs were bred to generate HOD x KEL mice. (B) KEL and HEL expression on WT and HOD x KEL RBC ghosts was determined by western blot analysis. (C) Expression of KEL and HEL was determined on WT and HOD x KEL RBCs by flow cytometry, both individually and by dual staining of HEL and KEL together. (D) Expression of KEL and HEL was determined on WT and HOD x KEL platelets by flow cytometry, both individually and by dual staining of HEL and KEL together. (E) Expression of KEL and HEL was determined on WT and HOD x KEL white blood cells (WBCs) by flow cytometry, both individually and by dual staining of HEL and KEL together.

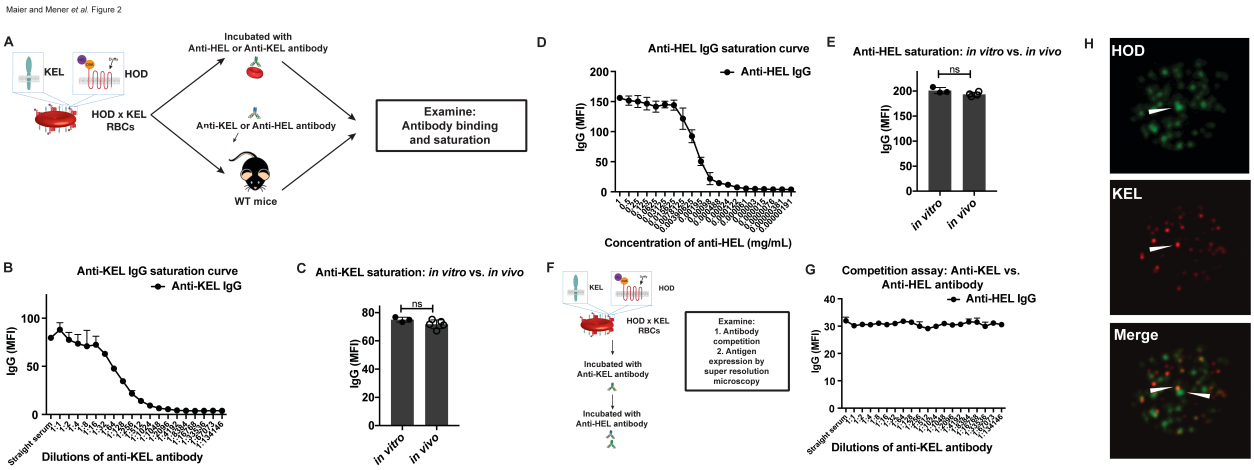

Figure 2: Saturation of HOD x KEL RBCs occurs with anti-HEL and anti-KEL antibodies *in vitro* and *in vivo*. (A) HOD x KEL RBCs were either incubated with anti-KEL or anti-HEL antibody, or transfused into WT mice previously immunized with anti-KEL or anti-HEL antibody. Antibody binding and saturation was determined. (B) Saturation of HOD x KEL RBCs by anti-KEL antibody was determined *in vitro* over a range of dilutions. (C) The serum from mice treated with anti-KEL antibody following transfusion with HOD x KEL RBCs was assessed for binding to HOD x KEL RBCs by flow crossmatch. Saturation of HOD x KEL RBCs by anti-KEL antibody was compared *in vitro* and *in vivo*. (D) Saturation of HOD x KEL RBCs by anti-HEL antibody was determined *in vitro* over a wide range of concentrations. (E) The serum from mice treated with anti-HEL antibody following transfusion with HOD x KEL RBCs was assessed for binding to HOD x KEL RBCs by flow crossmatch. Saturation of HOD x KEL RBCs by anti-HEL antibody was compared *in vitro* and *in vivo*. (F) HOD x KEL RBCs were incubated with anti-KEL antibody prior to incubation with anti-HEL antibody to determine competition between the antibodies. (G) Anti-HEL antibody binding to HOD x KEL RBCs was assessed over a wide range of anti-KEL antibody dilutions. (H), Anti-

HEL and anti-KEL binding to HOD x KEL RBCs was assessed by super resolution microscopy. For (C) and (E), ns = not significant by unpaired student's t-test.

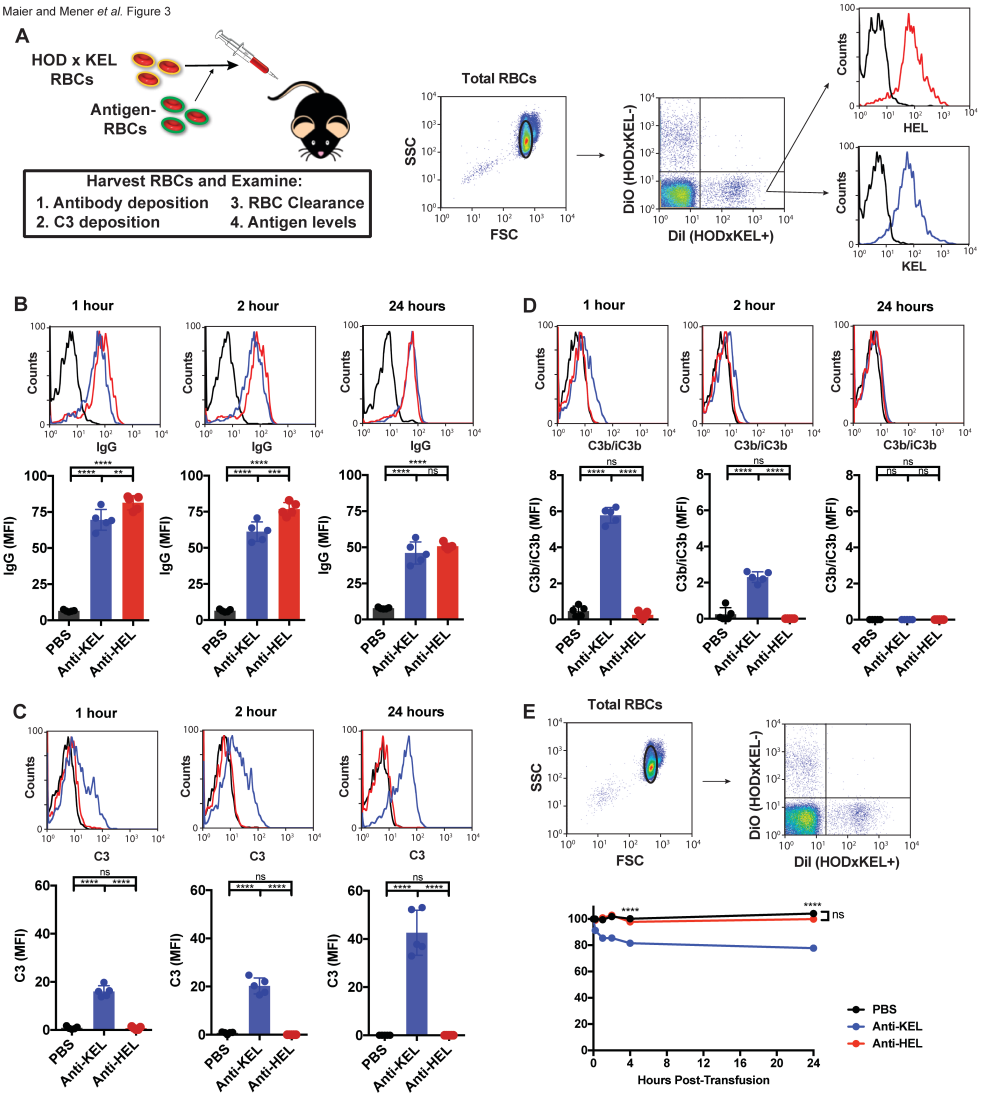

Figure 3: IgG antibody and complement deposition is detectable on HOD x KEL RBCs post-transfusion, with clearance of HOD x KEL RBCs occurring in mice treated immunized with anti-KEL antibody. (A) HOD x KEL RBCs were labeled prior to transfusion into PBS (no antibody), anti-KEL or anti-HEL treated WT mice. (B) Post-transfusion, RBCs were harvested and the level of IgG deposition was measured by a direct antiglobulin test in terms of mean fluorescence intensity (MFI) at 1 hour, 2 hours

and 24 hours post-transfusion. (C) Total C3 was measured on HOD x KEL RBCs at 1 hour, 2 hours and 24 hours post-transfusion and expressed in terms of mean fluorescence intensity (MFI). (D) Active complement (C3b/iC3b) was measured on HOD x KEL RBCs at 1 hour, 2 hours and 24 hours post-transfusion and expressed in terms of MFI. (E) Post-transfusion, total RBCs were harvested and the RBC survival was determined by directly comparing the ratio of HOD x KEL^+ to HOD x KEL^- RBCs. Means ± SD shown. For (A)-(D), ****=p<.0001, ***=p<.0006, **=p<.008, *=p<.05 and ns = not significant by One-way ANOVA with Tukey's multiple comparison test. For (E), ****=p<.0001 and ns = not significant by Two-way ANOVA with Dunnett's multiple comparisons test.

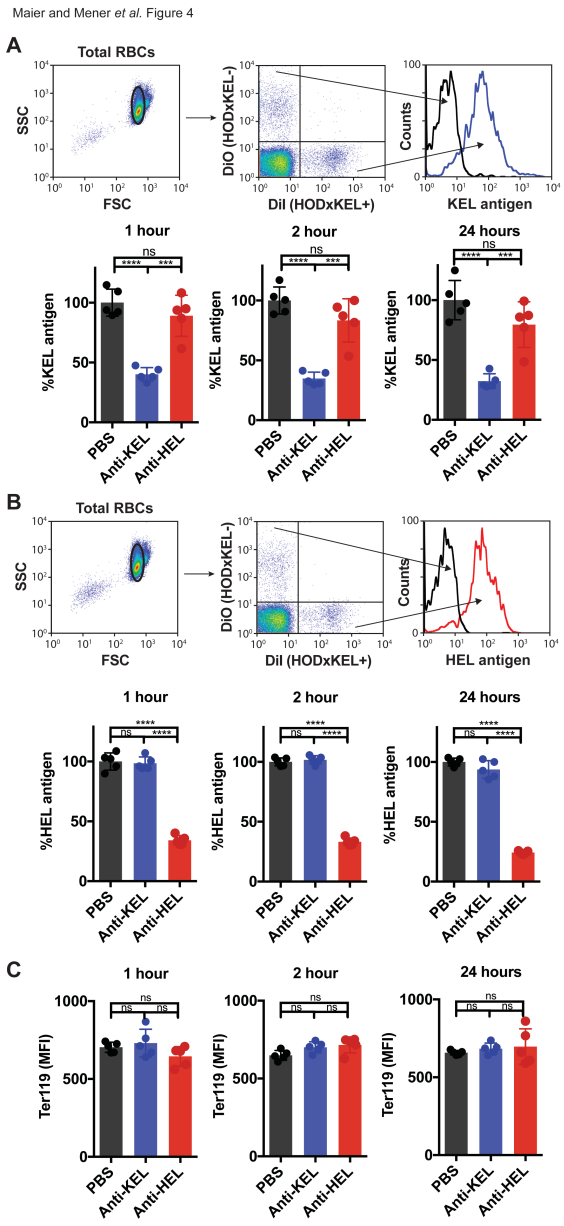

Figure 4: Antibody-induced decreases in the level of detectable antigen are antigen-specific. WT mice were treated with PBS (no antibody), anti-KEL or anti-HEL antibodies prior to exposure to labeled HOD x KEL RBCs. (A) Post-transfusion, HOD x KEL RBCs were stained for the level of detectable KEL antigen, which was measured as a percentage of KEL antigen normalized to KEL antigen in WT mice treated with PBS. (B) HOD x KEL RBCs were stained for the level of detectable HEL antigen, which was

measured as a percentage of HEL antigen normalized to HEL antigen in WT mice treated with PBS. (C) HOD x KEL RBCs were stained for the level of detectable Ter119 in terms of mean fluorescence intensity (MFI). Means ± SD shown. For (A)-(C), ***=p<.0002 and ****=p<.0001 and ns = not significant by One-way ANOVA with Tukey's multiple comparison test.

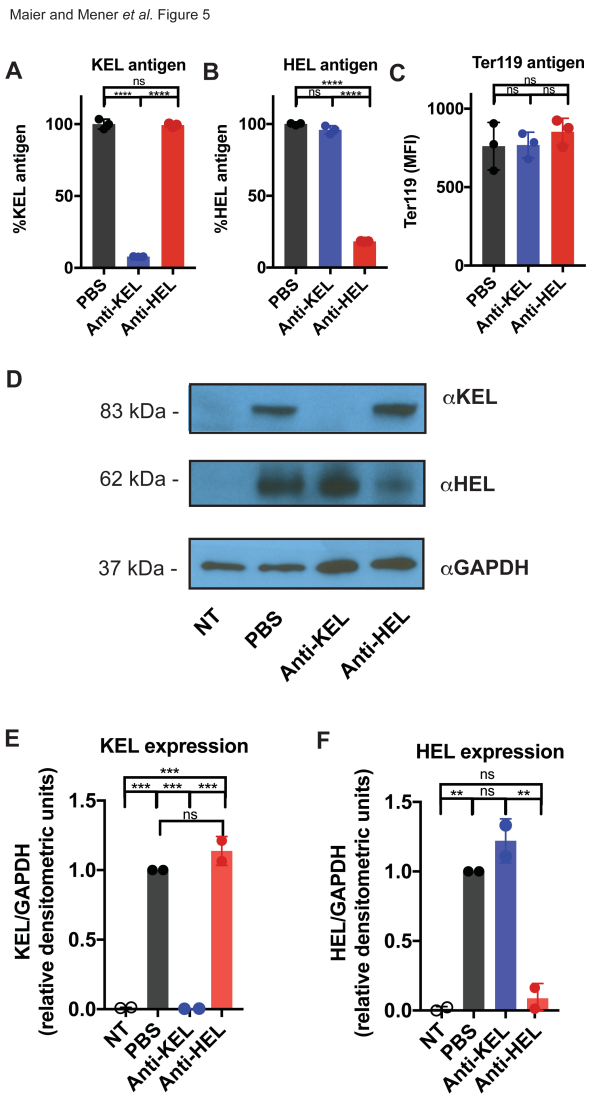

Figure 5: Antibody-induced decreases in the level of detectable antigen reflects antigen-specific removal. WT mice were treated with PBS (no antibody), anti-KEL or anti-HEL antibodies prior to exposure to labeled HOD x KEL RBCs. RBCs were tracked until decreases in the level of detectable KEL and HEL antigens occurred. (A) Post-transfusion, HOD x KEL RBCs were stained for the level of detectable KEL antigen, which was measured as a percentage of KEL antigen normalized to KEL antigen in WT mice treated with PBS. (B) HOD x KEL RBCs were stained for the level of detectable

HEL antigen, which was measured as a percentage of HEL antigen normalized to HEL antigen in WT mice treated with PBS. (C) HOD x KEL RBCs were stained for the level of detectable Ter119 antigen expressed in terms of MFI. (D) HOD x KEL RBC ghosts or ghosts from non-transfused mice (NT) were prepared and the level of antigen was investigated by western blot analysis by probing for KEL, HEL and GAPDH. (E)-(F), Densitometry was performed using ImageJ software and the relative densitometric units were assessed for KEL antigen (E) and HEL antigen (F). Means ± SD shown. For (A)-(C) and (E)-(F), ****=p<.0001, ***=p<.0002, **=p<.002 and ns = not significant by One-way ANOVA with Tukey's multiple comparison test.

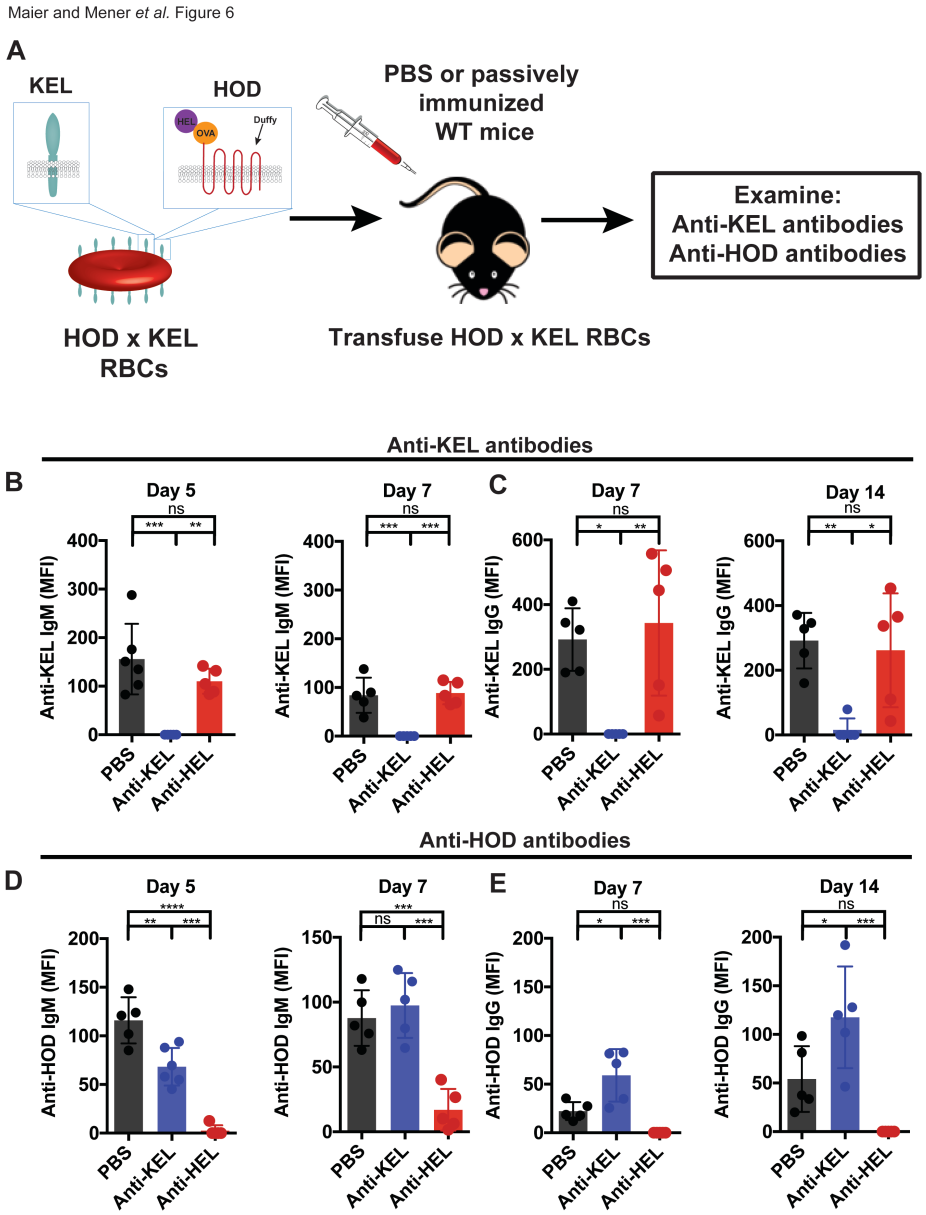

Figure 6: Exposure to antigen-specific antibodies induces antigen-specific antibody-mediated immunosuppression. (A) WT mice were treated either with PBS (no antibody), anti-KEL or anti-HEL antibodies prior to exposure to HOD x KEL RBCs. Anti-KEL IgM (B) and anti-KEL IgG (C) antibodies were examined by flow crossmatch and measured in terms of mean fluorescence intensity (MFI). Anti-HOD IgM (D) and anti-HOD IgG (E) antibodies were examined by flow crossmatch and measured in terms of MFI. Means ± SD shown. For (B)-(E), ****=p<.0001, ***=p<.0009, **=p<.007,

*=p<.05 and ns = not significant by One-way ANOVA with Tukey's multiple comparison test.

Chapter 4: Complement component 3 Negatively Regulates Antibody Response by Modulation of RBC Antigen

Abstract: Red blood cell (RBC) alloimmunization can make it difficult to procure compatible RBCs for future transfusion, directly leading to increased morbidity and mortality in transfusion-dependent patients. However, the factors that regulate RBC alloimmunization remain incompletely understood. As complement has been shown to serve as a key adjuvant in the development of antibody responses against microbes, we examined the impact of complement on RBC alloimmunization. In contrast to the impact of complement component 3 (C3) in the development of an immune response following microbial exposure, transfusion of C3 knockout (C3 KO) recipients with RBCs expressing KEL (KEL RBCs) actually resulted in an enhanced anti-KEL antibody response. The impact of C3 appeared to be specific to KEL, as transfusion of RBCs bearing another model antigen, the chimeric HOD antigen (HEL, OVA and Duffy), into C3 KO recipients failed to result in a similar increase in antibody formation. KEL RBCs experienced enhanced C3 deposition and loss of detectable target antigen over time when compared to HOD RBCs, suggesting that C3 may inhibit antibody formation by impacting the accessibility of the target KEL antigen. Loss of detectable KEL on the RBC surface did not reflect antigen masking by C3, but instead appeared to result from actual removal of the KEL antigen, as western blot analysis demonstrated complete loss of detectable KEL protein. Consistent with this, exposure of wild type B6 or C3 KO recipients to KEL RBCs with reduced levels of detectable KEL antigen resulted in a significantly reduced anti-KEL antibody response. These results suggest that C3 possesses a unique ability to actually suppress antibody formation following transfusion by reducing the availability of the target antigen on the RBC surface.

Introduction:

Red blood cell (RBC) transfusion therapy can provide a life-saving intervention for patients with congenital hemoglobinopathies or general bone marrow failure syndromes (1-5). However, while RBC transfusion can be beneficial in a variety of patient populations, transfusion is not without risk. Patients who receive chronic transfusions are prone to developing alloantibodies against RBC alloantigens that differ between RBC donors and recipients (6, 7). Currently, the only method to reduce the development of alloantibodies against RBC antigens is to match donor and recipient for the common antigen targets of RBC alloimmunization. However, despite alloantigen matching protocols, 30-50% of chronically transfused patients can still become alloimmunized (5, 8, 9). The development of alloantibodies against various RBC antigens can make it difficult to find compatible blood for future transfusions (8, 9). Furthermore, individuals who develop alloantibodies are at an increased risk of developing hemolytic transfusion reactions (10-12), one of the most common causes of transfusion-related mortality (5, 13). As no current prophylactic measure exists that can actively inhibit RBC alloimmunization, a greater understanding of the molecular mechanisms that govern RBC alloantibody development is needed.

Previous studies demonstrate that in the absence of complement, little detectable humoral immunity can be observed following microbial challenge in mice (14-19). These results suggest that complement enhances the ability of hosts to drive an effective adaptive immune response, providing a key example of the intimate association between innate and adaptive immunity (15, 16). Previous studies also demonstrate that early

alloantibodies directed against RBC alloantigens can fix complement (20-25), suggesting that complement fixation on the RBC surface may likewise favorably impact the development of alloantibodies against RBC alloantigens. However, in contrast to the well-established role of complement in the development of a humoral immune response to various microbes (14, 17, 19, 26, 27), the impact of complement fixation on the surface of transfused RBCs on the development of a humoral immune response to a RBC alloantigen remains incompletely understood. The lack of studies examining the consequence of complement deposition on RBCs following RBC transfusion on subsequent adaptive immunity, in part, reflects the fact that unlike solid organ transplantation, RBCs from different strains of mice do not inherently possess distinct antigenic differences known to routinely induce alloantibodies following transfusion between strains (28, 29). In contrast, alternative approaches employing RBCs from different species does result in an immune response. However, as xenograft transfusion of rat and guinea pig RBCs results in rapid RBC clearance and an inflammatory response that is likely not consistent with immune recognition observed between RBC donor and recipient clinically (30-32), models that more accurately recapitulate key features of RBC alloimmunization are needed.

In an effort to overcome limitations in the study of factors that regulate immune responses to distinct alloantigens on the surface of RBCs isolated from the same species, several murine models have been recently developed. Of all of these models, the KEL model system, which employs a β-globin promoter to drive expression of the clinically relevant human KEL antigen specifically on murine RBCs (KEL RBCs), currently

provides the most compelling model capable of recapitulating key clinical features of RBC alloimmunization, including the ability to induce antibodies capable of clearing transfused RBCs (33-36). Using this model system, we investigated the consequences of complement deposition following RBC transfusion. We found that in contrast to the impact of complement on the development of a humoral immune response to microbes (14-19), transfusion of KEL RBCs into complement component 3 knockout (C3 KO) recipients actually resulted in an increased antibody response to KEL. These results suggest that unlike microbes, C3 can actually play an inhibitory role in the development of antibodies to transfused KEL RBCs and indicates a unique role for C3 in regulating adaptive immune responses directed against self.

Materials and Methods:

Mice

Female C57BL/6 (B6) mice were purchased from National Cancer Institute (Bethesda, MD). B6;129S4-C3tm1Crr/J (C3 KO) mice and C57BL/6N-*Hc*$^{tm1a(EUCOMM)Wtsi}$/J (C5 KO) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Fcer1g mice, deficient in Fcγ receptors I, III and IV (FcγR KO), were purchased from Taconic Laboratories (Hudson, NY). Donor KEL and HOD transgenic mice, expressing only KEL or HOD on RBCs under the γ-globin promoter, were generated as previously described (33, 34, 37), and were a generous gift from Dr. James Zimring (Bloodworks Northwest). All recipient and donor mice are on a C57BL/6 background and carry the $H2^b$ haplotype. All mice were used at 8-12 weeks of age, and were bred and housed by Emory Animal Resources in accordance to policies outlined by the Institutional Animal Care and Use Committee. All experiments included three to five mice per group and were repeated at least three times.

Mouse genotyping and screening

To confirm the genetic deletion of C3 in the C3 KO mice, DNA was isolated from peripheral blood using the DNeasy Blood and Tissue Kit (QIAGEN). Polymerase chain reaction (PCR) was performed using Taq Polymerase Master Mix Red (Apex) with primer sequences provided by The Jackson Laboratory (common primer olMR1325 sequence: ATC TTG AGT GCA CCA AGC C, wild-type primer olMR1326 sequence:

GGT TGC AGC AGT CTA TGA AGG and mutant primer olMR7415 sequence: GCC AGA GGC CAC TTG TGT AG). To confirm the genetic deletion of activating FcγRs in FcγR KO mice, peripheral blood was collected from B6 and FcγR KO mice into the anticoagulant acid citrate dextrose (ACD; Vacutainer, BD bioscience), followed by RBC lysis 3x for 15 minutes each with 150 μL RBC Lysing Buffer Hybri-Max (Sigma, St. Louis, MO). Following RBC lysis, peripheral lymphocytes were stained with anti-CD64 [(clone: X54-5/7.1), BioLegend, San Diego, CA] diluted 1:100 in flow cytometry staining buffer, FACS buffer [0.1% BSA (bovine serum albumin) in phosphate buffered saline (PBS)], for 30 minutes at 4°C. To confirm KEL expression on KEL RBCs, peripheral blood was collected from KEL mice into ACD, washed 3x with FACS buffer and stained with anti-KEL antibodies [(anti-Kp^b, MIMA-9 and anti-Js^b, MIMA-8), Bioxcell West Lebanon, NH] diluted in FACS buffer 1:100 for 20 minutes at room temperature, as previously described (36), followed by incubation with anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in FACS buffer for 20 minutes at room temperature. To confirm HEL on HOD RBCs, peripheral blood was collected from HOD mice into ACD, washed 3x with FACS buffer and stained with anti-HEL monoclonal antibodies [(clones: 2F4 and 4B7, both IgG1), Bioxcell, West Lebanon, NH] diluted 1:100 in FACS buffer for 20 minutes at room temperature, followed by anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in FACS buffer for 20 minutes at room temperature. RBC staining was measured by a FACSCalibur flow cytometer. Flow cytometric data was acquired by CellQuest Pro and analyzed using FlowJo software.

Blood collection and transfusion

For alloimmunization experiments, KEL or HOD RBCs were collected into a 50 mL conical tube containing ACD and washed 3x in PBS. After buffy coat aspiration between washes, each mouse was transfused via the lateral tail vein with 50 μL packed KEL or HOD RBCs resuspended in 300 μL PBS. To evaluate KEL or HOD RBC clearance, antibody deposition, antigen levels and complement fixation at various time points post-transfusion, KEL or HOD RBCs were collected into ACD and washed 3x in PBS. Following collection and washes, KEL or HOD RBCs were labeled with Molecular Probes Cell Tracker CM-DiI (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate; Life Technologies, Carlsbad, CA) to enable differentiation of KEL or HOD RBCs from recipient RBCs post transfusion. Control KEL or HOD negative RBCs (B6) were likewise labeled with a different lipophilic dye, DiO (3,3'-dihexadecyloxacarbocyanine perchlorate), to provide an internal KEL or HOD antigen negative RBC control, as previously described (33, 35, 38, 39). Labeling was confirmed individually by a FACSCalibur flow cytometer prior to mixing and transfusion. Following washing 3x in PBS after labeling, DiI-KEL RBCs and DiO-KEL negative RBCs were mixed equally. DiI-HOD RBCs and DiO-HOD negative RBCs were also mixed equally. Each mouse was transfused with 50 μL packed DiI-KEL RBCs (1:1 with DiO-KEL negative RBCs) or packed 50 μL DiI-HOD RBCs (1:1 with DiO-HOD negative RBCs) resuspended in 300 μL PBS into the lateral tail vein(33, 38, 39).

Staining for flow cytometry

Following transfusion, peripheral blood was collected by retro-orbital bleeding of each mouse into ACD and washed 3x in PBS. IgM and IgG on the RBC surface was detected through the direct antiglobulin test using anti-mouse IgM and IgG (Jackson Immunoresearch) diluted 1:100 in FACS buffer. Complement was detected using rat anti-mouse biotinylated antibodies against an epitope within C3d (Cedarlane) or initial forms of C3b (C3b/iC3b) (Cedarlane), followed by streptavidin (BD). Then, peripheral blood was stained for the level of detectable KEL antigen using polyclonal anti-KEL antibody diluted 1:100 in FACS buffer and incubated for 20 minutes at room temperature, as done previously (34, 35, 38, 40). Peripheral blood was stained for the level of detectable HEL antigen using polyclonal anti-HEL antibody diluted 1:100 in FACS buffer for 20 minutes at room temperature (39). Stained RBCs were then washed 3x in FACS buffer and incubated with the secondary antibody anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in FACS buffer for 20 minutes at room temperature, as done previously (34, 35, 38, 39, 41). For staining of white blood cells (WBCs) to evaluate the specificity of KEL and HOD expression, spleens were isolated from HOD and KEL donor mice. After washing in PBS and lysis with Red Blood Cell Lysing Buffer (Sigma, St. Louis, MO), WBCs were further washed 2x in PBS prior to staining as done previously (42). For staining of platelets, HOD and KEL donor mice were exsanguinated into ACD and the peripheral blood was centrifuged at 80 *g* with 1:2 PBS to isolate platelet rich plasma, as done previously (41, 43). WBCs, platelets or RBCs were then stained with polyclonal anti-KEL antibody or polyclonal anti-HEL antibody diluted 1:100 in FACS buffer. Following washing in FACS buffer, cells were stained with anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in FACS buffer. After

washing in FACS buffer, cells were stained with anti-CD45 (BD bioscience) for WBCs, anti-CD41 (BD bioscience) for platelets or anti-Ter119 (BD bioscience) for RBCs. Stained RBCs were then washed 3x in FACS buffer and diluted to a final total volume of 100 μL in FACS buffer.

Flow cytometry

After staining, 50 μL of each set of stained cells in FACS buffer was then added to 400 μL of FACS buffer and the level of detectable antigen, antibody bound or complement deposition was measured by a FACSCalibur flow cytometer by gating specifically on RBCs (Supplemental Figure S1). Data acquisition was accomplished by CellQuest Pro and was analyzed using FlowJo software (34, 35, 38). Mean fluorescence intensity (MFI) was used to assess the levels of detectable antigen, antibody bound or complement deposition. For the level of detectable antigen, the MFI of experimental mice was expressed as a percentage of the MFI of DiI-KEL RBCs transfused into KEL mice or DiI-HOD RBCs transfused into HOD mice.

Seroanalysis

To detect anti-KEL or anti-HOD alloantibody development in the serum, a flow cross-match was performed as previously described (33, 34, 41, 42, 44). Briefly, 10 μL of serum was incubated for 15 minutes at room temperature with 3 μL of either KEL or HOD RBCs. RBCs were then washed 3x in FACS buffer, followed by incubation with anti-mouse IgM or IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in FACS buffer for 30 minutes at room temperature. Non-specific background binding was

accounted for through incubation of serum from alloimmunized mice with antigen-negative RBCs. Alloantibody development through binding of serum alloantibodies to the antigen-positive RBCs was measured by FACSCalibur flow cytometry and analyzed using FlowJo software. Antibody development was assessed in terms of MFI as outlined previously (34, 41, 44). While the antibodies detected following HOD or KEL RBC transfusion are not technically "alloantibodies", they have been commonly referred to as alloantibodies in previous work, as the KEL and HOD systems are models of RBC alloimmunization (33, 34, 41, 44). In an effort to continue to provide uniformity of nomenclature within the field, we will continue to use this term to refer to antibodies generated in response to KEL or HOD RBC transfusion in the present work.

Western blot analysis

B6 mice were passively immunized with polyclonal anti-KEL antibody or injected with PBS, followed by transfusion with DiI-labeled KEL RBCs, as outlined previously (35). At day 1 post-transfusion, RBCs from immunized or non-immunized B6 mice were collected into ACD and washed 3x in PBS. Following washes, 100 μL of RBCs was incubated with 100 μL of protease inhibitor cocktail (Sigma) and 10 mL of RBC lysis buffer (5 mmoL/L sodium phosphate, pH 7.5). 1 mL aliquots of lysed RBCs were washed and centrifuged at 14,000 x g for 10 minutes until RBC membranes were transparent as done previously (44, 45). Following lysis and washes, membranes were re-suspended in 75 μL of 1x PBS with 25 μL of NuPage LDS Sample Buffer (4x) with 2.5% β-mercaptoethanol. Samples were heated at 70°C for 10 minutes then run on a reducing SDS-PAGE gel. Following transfer, the membrane was blocked in 5% low-fat milk, then

incubated overnight at 4°C in primary, polyclonal anti-KEL antibody (Abcam, clone: MM0435-12X3) diluted 1:250 in blocking buffer, as previously shown for detection of KEL (36, 40), or anti-GAPDH (Thermo Scientific, clone: GA1R) diluted 1:10,000 in blocking buffer for detection of GAPDH. Membranes were incubated in horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 (Bethyl Laboratories, Montgomery, TX) diluted 1:10,000 in blocking buffer.

Statistical analysis

Flow cytometry data was analyzed by FlowJo software and statistical analyses were performed in GraphPad Prism. For comparisons between two groups, we utilized the unpaired student's t-test. For groups of three or more, we used One-way ANOVA analysis with multiple comparisons performed by Tukey's post-test, unless otherwise noted. $p<.05$ was the cut-off for significance.

Results:

Antibody and C3 specifically deposit on KEL RBCs post-transfusion

To define the impact of C3 on antibody formation following RBC transfusion, we first sought to determine whether antibody deposition and complement fixation occurs following transfusion of KEL RBCs. To accomplish this, we first labeled packed KEL RBCs with a lipophilic dye, DiI, prior to transfusion to facilitate flow cytometric detection at various time points post-transfusion (Fig. 1A). To determine the specificity of potential antibody and complement interactions with KEL RBCs, RBCs that do not express KEL were labeled with a fluorescently distinct lipophilic dye, DiO, followed by co-transfusion with KEL RBCs into each recipient (Fig. 1A). This approach allows specific detection of each RBC population as a distinct population with the predicted antigen expression following transfusion (Fig 1A, Supplemental Figures S1-2). While no antibody could initially be detected on the surface of transfused KEL RBCs, by day 5, statistically significant IgM could be observed on the surface of KEL RBCs (Fig. 1B). These antibodies gradually increased over time and switched from primarily IgM deposition to IgG by day 21 post-transfusion (Fig. 1C), suggesting that the antibodies that form in response to KEL RBC transfusion possess the capacity to specifically engage the KEL RBC target *in vivo* during the developing immune response. Antibody engagement appeared to be specific to KEL RBCs, as KEL negative RBCs circulating in the same recipients failed to exhibit significant antibody deposition (Fig. 1B-C). Together, these data indicate that transfusion of KEL RBCs appears to result in formation of anti-KEL antibodies that can specifically engage KEL RBCs.

Given the ability of antibodies to specifically engage KEL RBCs (Fig. 1), we next determined whether antibody engagement results in the deposition of complement during the developing immune response. Previous studies have demonstrated that antibody-mediated complement deposition first occurs as a cleavage product of C3 to C3b, which covalently attaches to the cell surface, but can be quickly degraded into the complement split product, C3d, that remains covalently attached to the cell surface (46-49); C3d is the common covalently attached complement target evaluated clinically when complement-mediated processes on the RBC surface are suspected (25). As a result, we not only examined early complement deposition following antibody engagement, but also determined the relative amount of early versus degraded C3 on KEL RBCs over time. As no anti-C3 antibody is currently available that can specifically differentiate C3d from total C3 (given that C3d is part of the entire C3 protein), early versus degraded complement detection can be accomplished by examining cells for epitopes of C3b and iC3b that are removed following degradation to C3d and comparing this to total C3 using an antibody that recognizes an epitope within C3d (35). Following transfusion, total C3 could be readily detected on the surface of KEL RBCs (Fig. 1D). In contrast, very little C3b could be detected on the cell surface at any time point evaluated (Supplemental Fig. S3A-D), suggesting that complement activated on the KEL RBC surface during the development of an immune response rapidly degrades to C3d. No C3 could be detected on the surface of KEL negative RBCs co-transfused with KEL RBCs (Fig. 1D), suggesting that anti-KEL antibody engagement specifically occurred on KEL RBCs and that this appears to in turn result in KEL RBC-specific C3 deposition. Taken together,

these results demonstrate that antibodies that form in response to KEL RBC transfusion not only possess the ability to specifically engage KEL RBCs, but also can fix complement.

C3 KO recipients exhibit an increased immune response to transfused KEL RBCs

Given the impact of C3 on developing immune responses following microbial challenge and the ability of antibodies that develop in response to KEL RBC transfusion to fix complement (14-19), we next sought to directly examine the potential impact of complement on the immune response following KEL RBC transfusion. To accomplish this, we transfused B6 or C3 KO recipients with KEL RBCs, followed by an evaluation of anti-KEL antibody formation over time (Fig. 2A). While KEL RBCs induced anti-KEL antibodies in B6 recipients, as seen previously (33, 35, 45), similar exposure in C3 KO recipients unexpectedly resulted in a statistically significant increase in IgM anti-KEL antibody formation by day 5 post-exposure (Fig. 2B). Similar increases in IgG anti-KEL antibodies were also observed in C3 KO recipients at day 21 post-exposure (Fig. 2B). To determine whether the increased antibody response observed in C3 KO recipients was specific to KEL RBCs, we next determined whether RBCs expressing an entirely different model RBC alloantigen, the HOD (hen egg lysozyme (HEL), ovalbumin (OVA) and human Duffy) antigen, likewise induced an enhanced immune response following transfusion into C3 KO recipients. In contrast to the increased anti-KEL antibody response observed following transfusion of KEL RBCs into C3 KO recipients (Fig. 2B), transfusion of C3 KO recipients with HOD RBCs failed to result in increased anti-HOD IgM or IgG (Fig. 2C). The ability of KEL RBCs to induce an enhanced anti-KEL

antibody response stands in stark contrast to previous studies implicating a key requirement for C3 in the development of antibodies following pathogen exposure (14, 19, 26, 50-54). Instead, these results suggest that C3 may actually play an inhibitory role when antibodies develop against the KEL RBC alloantigen following KEL RBC transfusion.

Complement facilitates loss of detectable KEL antigen independent of changes in RBC clearance

Given the impact of C3 on anti-KEL antibody formation specifically, we first sought to determine whether differences in C3 deposition on the RBC surface might correlate with differences in antibody formation observed following KEL or HOD RBC transfusion into B6 versus C3 KO recipients. This is especially important when considering that previous studies suggest that antibody engagement of different RBC antigens can differentially impact the likelihood of complement activation (25). While total C3 could be readily detected specifically on the surface of KEL RBCs, significantly less complement could be detected on HOD RBCs when evaluated in parallel (Fig. 3A). Importantly, transfusion of HOD or KEL RBCs into C3 KO recipients failed to result in detectable C3 deposition (Fig. 3B), which demonstrated that the detection of C3 was likely specific. Furthermore, differences in C3 deposition did not appear to reflect alterations in the level of initial antibody engagement, as the level of detectable antibody on the surface of KEL RBCs or HOD RBCs appeared to be very similar (Fig. 4). While a trend toward increased IgG1, IgG2b and IgG3 antibodies following KEL RBC transfusion was observed compared to HOD RBC-induced antibody formation, these differenes failed to reach statistical

significance (Supplemental Figure S3). Taken together, these results demonstrate that antibodies that form in response to both KEL and HOD RBC transfusion can fix complement. However, differences in the level of C3 deposition on KEL and HOD RBCs may impact the ability of C3 to regulate alloantibody formation specifically following KEL RBC transfusion.

The differential complement deposition on the RBC surface of KEL and HOD RBCs, coupled with previous studies suggesting that complement may potentially impact the availability of the target antigen (55), suggests that complement may physically mask or otherwise alter the availability of the KEL antigen to the ongoing immune response. This in turn would be predicted to impact the ongoing anti-KEL immune response. To test this, we next determined the consequence of antibody engagement and C3 deposition on KEL antigen availability on the KEL RBC surface over time. As a control, we also transfused KEL RBCs into KEL donor mice, which do not generate anti-KEL antibodies (33). This allows parallel evaluation of the overall stability of the KEL antigen over time following transfusion in the absence of an immune response. While the KEL antigen could be readily detected initially following transfusion into KEL, B6 or C3 KO mice, consistent with the possibility that C3 may actually regulate KEL antigen accessibility during the developing immune response, transfusion of KEL RBCs into B6 recipients actually resulted in decreased levels of detectable KEL antigen over time when compared to control KEL mice transfused with syngeneic KEL RBCs (Fig. 5A). This decrease in the level of detectable KEL antigen in B6 recipients also correlated with the development of anti-KEL antibodies (Fig. 2B). In contrast, a similar decrease in the level of detectable

KEL antigen over time failed to occur at the same rate in C3 KO recipients, suggesting that while antibody itself may limit accessibility to the KEL antigen, C3 clearly accelerates this process (Fig. 5A). To determine the specificity of C3-dependent changes to the KEL antigen over time, given the inability of C3 to regulate HOD RBC-induced antibody formation, we next evaluated the potential impact of C3 on the HOD antigen following transfusion. In contrast to KEL RBCs, alterations in the levels of detectable HEL antigen over time did not differ following transfusion into B6 or C3 KO mice (Fig. 5B). Taken together, these results suggest that C3 may negatively regulate immunity toward KEL by impacting the availability of the KEL antigen on the KEL RBC surface.

To control for the possibility that complement may accelerate RBC removal and therefore impact KEL RBC immunogenicity independent of C3-induced alterations in the levels of detectable antigen on the RBC surface, we next sought to determine whether C3 impacts KEL RBC removal during the developing anti-KEL immune response. To accomplish this, labeled DiI-KEL RBCs were transfused into B6 and C3 KO recipients and the relative rate of KEL RBC removal compared to DiO-KEL negative RBCs co-transfused with KEL RBCs was determined over time (Fig. 6A). Consistent with the lack of detectable active complement on the cell surface (Supplemental Fig. S4A-D) (25, 35, 56, 57), no increase in KEL RBC clearance was observed in B6 mice as compared to C3 KO recipients (Fig. 6B), suggesting that C3 does not appear to induce detectable increases in the clearance of KEL RBCs following transfusion. Similarly, no differences in HOD RBC clearance were observed between B6 and C3 KO recipients (Fig. 6C-D). These

results suggest that while C3 can be deposited on the RBC surface, it does not appear to impact KEL or HOD RBC removal.

To assess whether other immune factors, independent of C3, may regulate KEL antigen availability on KEL RBCs post-transfusion over time, we examined the potential impact of C5, a downstream complement effector from C3 (58). Transfused KEL RBCs in C5 KO recipients were then assessed for the level of detectable KEL antigen compared to KEL RBCs transfused into syngeneic KEL RBC recipients. In contrast to the impact of C3 on the accessibility of the KEL antigen on KEL RBCs, transfusion of KEL RBCs into C5 KO mice resulted in a similar decrease in detectable KEL antigen levels when compared to B6 mice (Fig. 7A), suggesting that the downstream complement effector, C5, is not required for alterations in detectable KEL antigen levels. As previous studies suggest that Fcγ receptors (FcγR) may also possess the ability to impact antigen accessibility (38, 39, 59), we next examined the potential impact of activating FcγRs on KEL antigen availability using the common gamma chain KO mouse (FcγR KO), as done previously (38, 59). However, similar to C5 KO recipients, no difference in KEL antigen could be detected when comparing KEL RBCs following transfusion into B6 or FcγR KO recipients (Fig. 7B). Given the lack of alterations in KEL antigen observed in C5 KO or FcγR KO recipients, we next examined whether transfusion of KEL RBCs into C5 KO or FcγR KO recipients impacts the anti-KEL antibody response. Transfusion of KEL RBCs into C5 KO and FcγR KO mice failed to result in altered levels of anti-KEL IgM or IgG when compared to B6 mice (Fig. 8A-B), suggesting that while C3 plays an inhibitory role

in the antibody response to KEL, C5 and FcγRs do not appear to negatively or positively impact antigen levels or anti-KEL antibody formation following KEL RBC transfusion.

Antibody-induced antigen changes on the RBC surface impact antibody response

The results thus far suggest that antibody-induced deposition of complement on KEL RBCs impacts the availability of the cell surface KEL antigen, which in turn may reduce immune detection and therefore the magnitude of the ongoing anti-KEL immune response. In order to directly examine the consequence of alterations in the levels of detectable KEL antigen on the development of an anti-KEL immune response, we next sought to evaluate the impact of reduced KEL antigen availability on the development of anti-KEL antibodies following KEL RBC transfusion. To accomplish this, we first induced alterations to the KEL antigen on KEL RBCs in KEL donors prior to RBC isolation and transfusion into separate recipients by directly injecting anti-KEL antibodies into KEL RBC donors (Fig. 9A). Injection of anti-KEL antibodies in this manner resulted in rapid antibody engagement and complement deposition on KEL RBCs (Fig. 9B). The level of detectable KEL antigen likewise decreased to approximately 50% of the initial values (Fig. 9B), providing a unique KEL RBC substrate to directly test the impact of reduced KEL availability on the ability of KEL RBCs to induce antibody formation. As a result, we next transferred KEL RBCs from anti-KEL antibody treated or non-treated KEL donors into B6 or C3 KO recipients. Consistent with the possibility that reduced KEL antigen availability may impact anti-KEL antibody formation, B6 and C3 KO recipients that received KEL RBCs with decreased KEL antigen failed to develop a significant anti-KEL antibody response in either B6 or C3 KO mice (Fig. 9C-D), while

unaltered KEL RBCs induced a robust antibody response when evaluated in parallel. These results suggest that reductions in the level of detectable KEL on the KEL RBC surface can significantly impact the development of an anti-KEL antibody response.

Next, we sought to determine whether loss of detectable KEL antigen reflects masking of the KEL antigen or removal from the KEL RBC surface following allogeneic KEL RBC transfusion. To accomplish this, we injected B6 mice with polyclonal anti-KEL antibody prior to exposure to KEL RBCs, followed by RBC harvest 24 hours following KEL RBC transfusion into these immunized or non immunized recipients (Fig. 9E). KEL RBCs were then evaluted for the level of detectable KEL antigen on transfused KEL RBCs first by flow cytometry. Transfusion of KEL RBCs into immunized recipients resulted in a near to complete loss of detectable KEL antigen, as detected by flow cytometry 24 hours following injection (Fig. 9F). To determine whether loss of KEL antigen in this setting reflected loss or simple masking of KEL, KEL RBCs transfused into immunized or non-immunized recipients were then subjected to western blot analysis. While KEL could be readily detected in B6 recipients injected with KEL RBCs in the absence of anti-KEL antibodies (non-immunized), no KEL could be detected in anti-KEL immunized recipients (Fig. 9G). These results suggest that the loss of detectable KEL, as measured by flow cytometry, likely reflects actual removal of the KEL antigen from KEL RBCs. In each of these situations, the loss of KEL antigen appeared to be specific, as similar changes in the RBC specific cell surface marker, Ter119, failed to similarly occur (Fig. 9H). Importantly, loss of detectable KEL antigen on transfused KEL RBCs was not due to significant KEL RBC clearance, and therefore a simple reduction in KEL RBC

numbers, as KEL RBCs were transfused at a pre-adjusted ratio to ensure that the final KEL RBC percentage at the time of western blot analysis were the same (Fig. 9I). These results suggest that complement appears to facilitate removal of the KEL antigen, thereby reducing the availability of KEL for the ongoing anti-KEL immune response, while also leaving the remaining KEL RBCs intact.

Discussion:

The ability of C3 to negatively impact the anti-KEL antibody response reveals an unexpected role for C3 in regulating antibody formation against an antigen expressed on transfused RBCs. Previous studies demonstrate a critical role for C3 in the development of antibodies toward a wide variety of microbes (14-19). Unlike microbes, RBCs express key complement regulatory proteins, including CD55 and CD59, which regulate complement activation by interfering with complement assembly and effector function (60). While the exact nature of complement regulation can vary between species, the general features that govern complement activation that evolved to protect self from complement are highly conserved and represent an evolutionarily ancient process (25, 61, 62). The results of the present study suggest that in addition to the differences in the outcome of complement activation on the surface of a RBC when compared to a microbe (25, 35), which can protect RBCs from complement-mediated injury and removal in the presence or absence of antibodies (35, 55, 63-65), complement also appears to possess the ability to negatively regulate the immune response toward an antigen on a RBC surface by impacting the availability of the target antigen for the ongoing immune response. Given the complexity of complement inactivation on the cell surface, in addition to many different complement receptors and cell populations that can engage C3 (66-69), the regulation of C3-mediated target antigen removal on the RBC surface may also reflect an equally complex and coordinated process. This process may have co-evolved with complement activation of adaptive immunity to actually protect hosts from undesirable immune responses once directed toward an antigen on a self-like surface, such as a transfused RBC. Thus, while protection of cells from complement effector

function represents a well-documented, evolutionarily conserved process with significant implications in human disease (63-65, 70, 71), the ability of complement to likewise negatively regulate antibody formation following engagement of RBCs provides a previously unrecognized additional ability of complement to differentially regulate adaptive immunity.

The ability of antibodies in general to suppress immune function represents a long recognized, yet poorly understood process previously suggested to reflect a key regulatory loop in preventing or reducing antibody formation (72, 73). While previous studies have associated antibody-mediated immunosuppression with complete removal of antigen positive cells, the development of anti-idiotype antibodies and a variety of other mechanisms (22, 74), the ability of complement to negatively regulate antibody formation specifically following KEL RBC transfusion represents a unique role of complement in the regulation of antibody formation. This is especially important when considering that unlike cellular immunity, where intimate contact between effector cells and host targets allows hosts to directly regulate immune activity (75, 76), once antibodies are released from antibody secreting cells, the target tissue is often spatially and temporally separated from the antibody secreting cell, making it difficult, if not impossible, for the target tissue to provide direct feedback to specific antibody secreting cells (77, 78). These data suggest that, along with the expression of complement regulatory proteins on RBCs to protect against complement effector functions (35, 55, 63-65), RBCs may have also evolved distinct mechanisms to directly regulate the impact of antibody binding in order to favorably inhibit the consequence of an undesirable

immune response when complement deposition does occur. As this process fails to occur following HOD RBC transfusion, these results also suggest that a threshold of C3 activation may be required for efficient C3-mediated regulation of an ongoing immune response. Thus, only when sufficient complement activation occurs does complement appear to be able to impact alloantibody formation. As recent studies suggest that RBCs with decreased levels of antigen may not possess the ability to induce antibodies despite equivalent levels of total antigen exposure (42), the presentation of antigen on the cell surface appears to be critical for effective immune recognition and response. Thus, C3-mediated antigen loss, even if not initially complete, may reduce KEL antigen levels below a threshold required to efficiently contribute to an ongoing immune response. The ability of complement to accelerate actual removal of the target antigen, while leaving the transfused RBCs intact, may therefore provide an additional layer of host protection against unwanted immunity.

Our results are consistent with previous studies and demonstrate that antibody engagement of RBC antigens does not uniformly result in similar levels of complement fixation (25, 30, 31, 40, 79). Characteristics of the RBC-bound antibody, including potential differences in IgG subclass levels, as well as inherent biochemical differences between RBC antigens themselves may influence the ability of antibody to preferentially induce complement fixation following antigen engagement. This may be especially apparent when considering that while a single IgM molecule can initiate complement deposition through engagement with C1q, two molecules of IgG must be bound in close proximity to similarly engage C1q (25, 30, 31, 80), suggesting that differences in antigen

density, lateral mobility and sites of antibody-antigen engagement may impact the relative ability of IgM and/or IgG antibodies to efficiently initiate complement activation following antibody binding (25, 81). The increased ability of IgM to activate complement may be particularly important in the setting of KEL and HOD RBC transfusion when considering that the maximum complement deposition on transfused KEL or HOD RBCs occurred 5 days post-transfusion when IgM anti-KEL or anti-HOD levels peak and virtually no detectable corresponding IgG anti-KEL or anti-HOD antibodies were present. Indeed, these results suggest that while IgG subclass could certainly impact predilections for C3 fixation following antibody-antigen engagement, unique antigen characteristics that differ between KEL and HOD may in part regulate the ability of IgM anti-KEL to more efficiently fix C3 on the RBC surface. This is especially important when considering that in contrast to the KEL antigen, which is a single membrane pass antigen, and therefore may possess greater lateral mobility within the RBC membrane (82), the HOD RBC antigen contains the seven transmembrane pass human antigen, Duffy (37, 83), which may not lend itself to the same level of optimal IgM engagement required for efficient C1q binding and subsequent C3 fixation. Although many studies in the past have recognized that certain antibody-antigen combinations differentially fix complement (25, 30, 31, 40), future studies will certainly be needed to determine the underlying mechanisms that dictate whether C3 fixation will occur following antibody engagement of a particular RBC antigen.

Given the evolutionary ancient role of C3 in providing direct immunity, in addition to regulating immune function (84), many different cell types interact with C3 through a

variety of complement receptors (CRs) (85). As RBCs pass through many organs, including splenic sinusoids, cells such as red pulp macrophages may phagocytose RBCs following antibody engagement and complement fixation (86, 87). Additional cells, such as other CR-bearing myeloid cells in the spleen, blood or other compartments, may likewise participate or primarily be responsible for this process (88). Given that there are many different immune populations that express various CRs, the processes that govern C3-mediated RBC clearance and antigen removal are also likely complex, and may involve multiple cell types and CRs. For example, while CR of the immunoglobulin family (CRIg) is a more recently described CR, CRIg may work in concert with the more classically described CRs 1 through 4 (68, 69, 89, 90), suggesting a cooperative role of CR function. This is especially important when considering that RBCs transverse the spleen and other vascular tissue, where a variety of CR-bearing immune populations reside, each of which can express distinct CRs and have been previously shown to facilitate immune complex removal (91-93). While CR can engage C3, they often display distinct preferences for various forms of C3 following activation (85, 94). Although bound forms of C3, such as C3b and iC3b, could not be detected on the surface of KEL RBCs, antibody-induced complement activation at the cell surface would be predicted to initially produce C3b, followed by iC3b, even if only transiently, which may in turn facilitate interactions between KEL RBCs and different CRs as KEL RBCs continue to circulate following antibody engagement (69, 95). As C5 did not appear to impact antigen levels or antibody formation, these results suggest that C5 or corresponding C5 receptors are not required for this process to occur. Although C3 and C5 are often the dominate players at key junctures in antibody-mediated complement activation, both with

respect to direct activation of downstream complement pathways and the engagement of receptors capable of mediating complement responses (58), these results do not rule out a potential role for C4 in this process. Finally, although FcγRs do not appear to be required for antigen loss in the setting of KEL RBC transfusion, they have been shown to be involved in the induction of alterations to target antigens in other settings (38, 59), suggesting that antibody engagement of different antibody effector systems may, in general, possess the capacity to impact antigen levels. Redundancy in antibody effector systems may therefore not only exist to aid in protection against microbial challenge, but also may serve as a mechanism to provide multiple avenues of protection against antibody-mediated injury to self. As CRs in particular are pleomorphic in function, removal of these receptors can result in a diverse range of phenotypes, only a part of which can be attributed to their role as CRs (69, 85). Therefore, understanding which receptor(s) may be involved in antigen removal, including the stage(s) in complement activation and degradation that may be responsible for this process, will be an important focus of future studies designed to determine how C3 facilitates antigen removal.

RBCs that express a single foreign and clinically relevant antigen not only aid in understanding key factors that may regulate RBC alloimmunization, but these models also provide a unique tool to understand the consequences of an antibody response in real time on target tissue. For many years, RBCs have provided an important substrate when seeking to study complement regulation and the consequences of antibody deposition on a host cell. Indeed, many of the seminal studies that describe key regulatory pathways of complement effector activity used RBCs as substrates when elucidating these pathways

(96-100). The inability of RBCs to divide and synthesize new antigen eliminates many of the confounding variables that would make examination of alternative self-like substrates difficult to study (38, 101). Despite the use of RBCs for decades to study key regulators of complement effector pathways on the cell surface, opportunities to similarly take advantage of RBCs to study the impact of complement deposition on the development of an immune response have not been equally available. This largely reflects the fact that murine RBCs isolated from different strains of mice do not express antigenic determinants capable of inducing an immune response (36). As a result, examining an immune response to RBCs using intraspecies RBCs has not been possible. While injection of RBCs from other species, such as sheep RBCs, results in a robust immune response and has been used for many years to study host immunity (102-106), sheep and other foreign RBCs are rapidly cleared (30), which can result in an artificial acceleration of an immune response that can compromise direct comparison of host regulation of complement outcomes during antibody development. Furthermore, interspecies complement regulators are often less effective at regulating complement (107, 108), likewise reducing the ability to directly examine the outcome of complement regulation in an otherwise syngeneic system. Chemical attachment of antigen to the cell surface damages RBCs (109, 110), and also results in rapid clearance and the production of an inflammatory response that prevents isolation of a single antigenic determinant on an otherwise normal cell as a distinct variable when seeking to determine the outcome of target antigen exposure on self. Thus, models of RBC alloimmunization provide an opportunity to take advantage of all the unique features of RBC biology, including the ability of RBCs to circulate in a homogenous fashion that allows this population to be

sampled over time, and thus examine in real time the impact of antibody formation on the RBC surface *in vivo*.

As in all experimental systems, limitations should be considered. Because our studies were performed in mice and not humans, our results may certainly inform our understanding of clinical phenomenon observed in humans, but this is not a direct model of human RBC alloimmunization. Furthermore, the KEL RBC model is also unique given that the RBCs between recipient and donor mice are otherwise syngeneic, except for the expression of the KEL RBC antigen. This is unlike transfused human RBCs, where in the absence of alloantigen matching, recipients may receive RBCs that differ in multiple RBC antigens from the donor, potentially leading to RBC alloimmunization. However, this reductionist model in mice allows for studying the direct contribution of immunologic factors that may govern the alloantibody response to KEL, which would not be ethically feasible nor logistically possible to study in a detailed fashion in humans. This is in part due to the fact that while RBC alloimmunization occurs clinically as a consequence of therapeutic RBC transfusion, intentionally inducing RBC alloimmunization to antigens outside of RhD is not ethical as it may put patients at risk for hemolytic transfusion reactions if emergent RBC transfusion is needed as only ABO and RhD antigens are routinely considered in the emergent setting. Therefore, despite the differences between humans and mice, this model system provides a unique opportunity to gain insight into potential variables that may govern alloimmunization. More specifically, our data presented here may provide further understanding of how complement may regulate the alloantibody response to RBC antigens through modulating

antigen on the RBC surface. This information may therefore provide the basis for future studies examining key determinants, such as complement, that may regulate an immune response following RBC transfusion.

Patients can develop an immune response following transfusion of various blood products, including the development of alloantibodies following exposure to distinct RBC alloantigens in the settings of therapeutic transfusion (9, 111). Similar to the outcome of this study, the development of antibodies against polymorphic alloantigens on a RBC surface in patients often not only results in antibody formation, but also complement deposition (112, 113). However, unlike in mice of identical genetic backgrounds, the immune response in patients following RBC alloantigen exposure can vary significantly. While a variety of factors likely contribute to this phenomenon (114, 115), the results of this murine study suggest that variability in the levels and activity of complement proteins may impact this process. This is especially important when considering that many disease states that can become indications for RBC transfusion often exhibit significant variability in complement levels and complement deposition on circulating RBCs (116, 117). Thus, alterations in complement engagement following antibody deposition during initial antibody development may influence the magnitude, and therefore the consequence of RBC alloantibody formation clinically. As a result, these studies not only provide fundamental insight into the role of complement in regulating antibody responses directed toward a target antigen expressed on a self-like RBC surface, but also likely have clinical implications in the development of RBC

alloantibodies, a process that significantly increases morbidity and mortality in patients who require repeat transfusion (8, 118).

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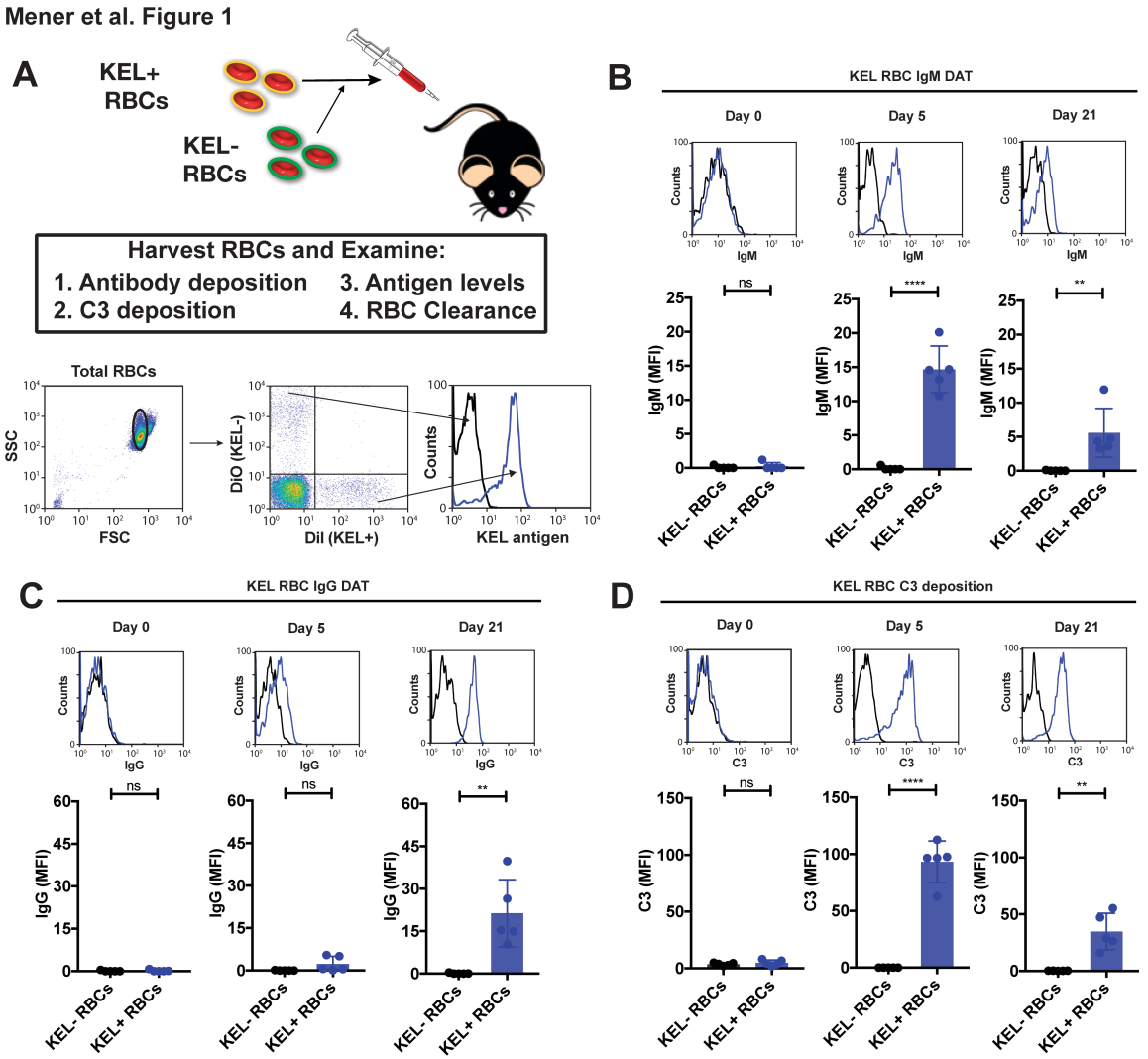


Figure 1: IgM, IgG and C3 specifically deposit on KEL positive RBCs post-transfusion. (**A**) Schematic overview of approach used to detect IgM, IgG and C3 on the surface of RBCs post-transfusion. Packed DiI-labeled KEL RBCs were co-transfused with packed DiO-labeled KEL negative B6 RBCs, followed by analysis of surface detectable IgM, IgG, C3 or antigen. Representative gating on DiI-labeled transfused KEL positive RBCs or DiO-labeled KEL negative RBCs with histogram analysis for the KEL antigen on each population. (**B**) KEL negative RBCs (KEL- RBCs) or KEL positive RBCs (KEL+ RBCs) were evaluated at days 0, 5 and 21 post-transfusion for deposition of IgM on the surface of RBCs by flow cytometry in B6 mice. (**C**) KEL negative RBCs

(KEL- RBCs) or KEL positive RBCs (KEL+ RBCs) were evaluated at days 0, 5 and 21 post-transfusion for deposition of IgG on the surface of RBCs by flow cytometry in B6 mice. (**D**) KEL negative RBCs (KEL- RBCs) or KEL positive RBCs (KEL+ RBCs) were evaluated at days 0, 5 and 21 post-transfusion for C3 deposition on the surface of RBCs by flow cytometry in B6 mice. For (**B-D**), ****=p<.0001, **=p<.01 and ns = not significant. Means ± s.d. shown.

Mener et al. Figure 2

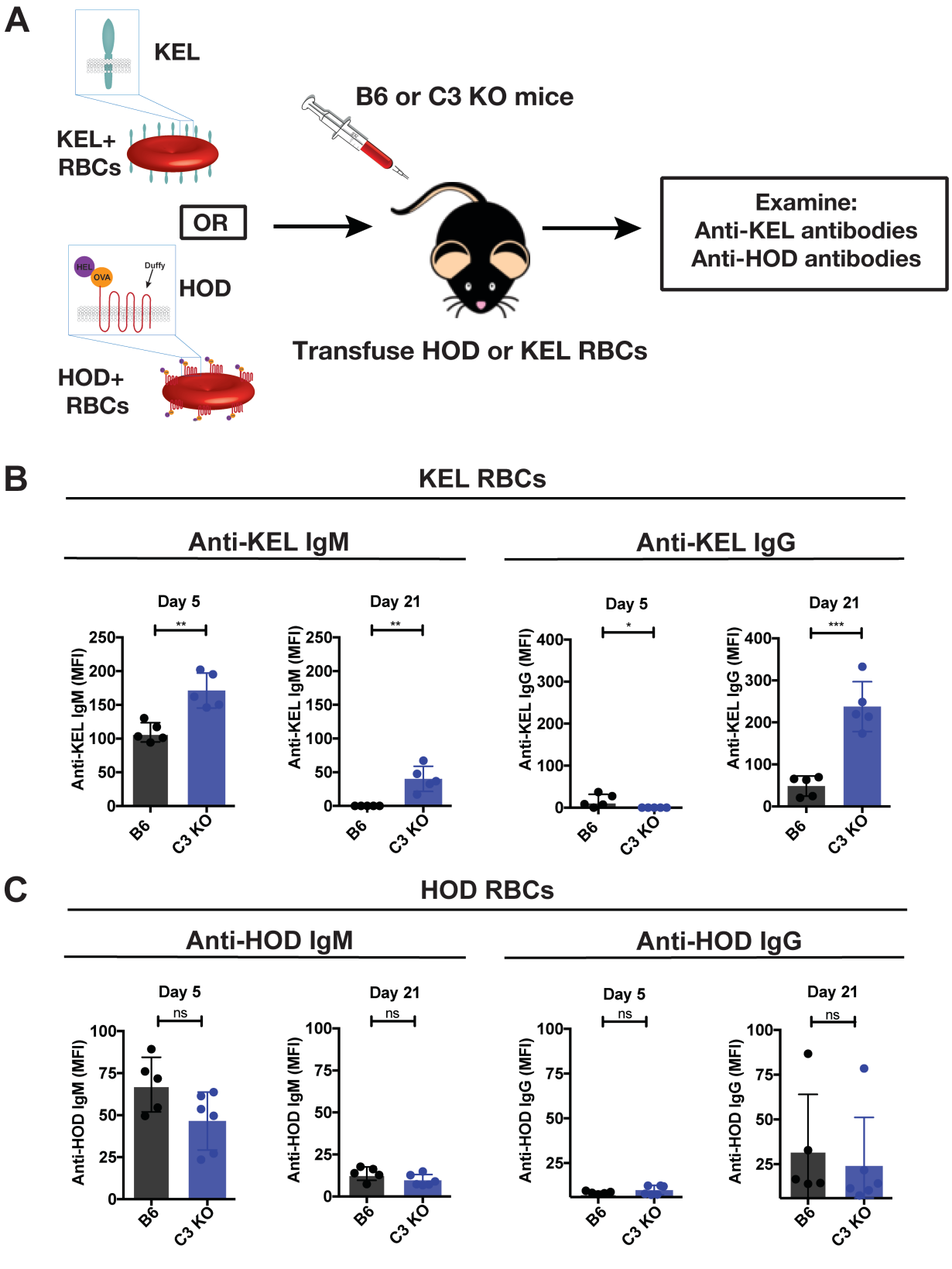


Figure 2: C3 KO recipients exhibit an increased anti-KEL antibody response following KEL RBC transfusion. (**A**) Schematic overview of KEL or HOD RBC transfusion into B6 and C3 KO mice, followed by examination of anti-KEL or anti-HOD antibodies, respectively. (**B**) Following exposure to KEL RBCs, serum from B6 or C3 KO recipients was assessed for development of anti-KEL IgM and IgG at days 5 and 21, respectively, following transfusion by flow cross-match. (**C**) Following exposure to HOD RBCs, serum from B6 or C3 KO recipients exposed to HOD RBCs was assessed for development of anti-HOD IgM and IgG at days 5 and 21, respectively, post-transfusion by flow cross-match. For (**B-C**), ***=p<.0002, **=p<.004, *=p<.05 and ns = not significant. Means ± s.d. shown.

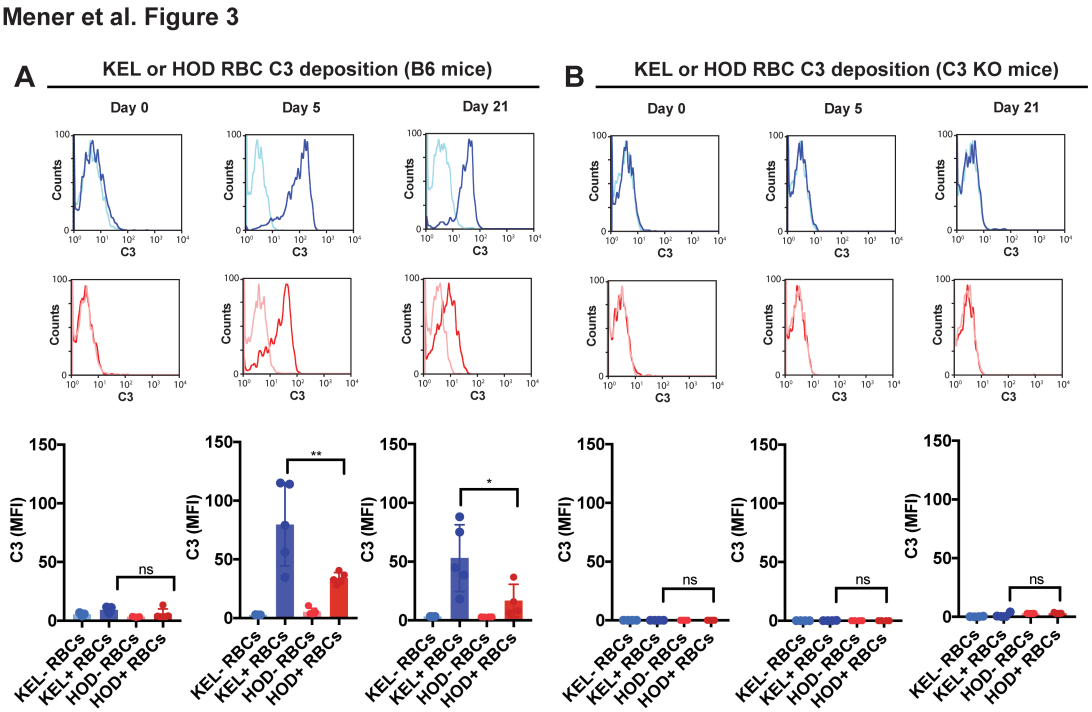


Figure 3: KEL RBCs exhibit increased levels of C3 deposition when compared to HOD RBCs over time post-transfusion. (**A**) Total C3 bound to circulating KEL negative RBCs (KEL- RBCs), KEL positive RBCs (KEL+ RBCs), HOD negative RBCs (HOD- RBCs) or HOD positive RBCs (HOD+ RBCs) was assessed on days 0, 5 and 21 post-transfusion into B6 mice. (**B**) Total C3 bound to circulating KEL negative RBCs (KEL- RBCs), KEL positive RBCs (KEL+ RBCs), HOD negative RBCs (HOD- RBCs) or HOD positive RBCs (HOD+ RBCs) were assessed on days 0, 5 and 21 post-transfusion into C3 KO mice. For (**A-B**), **=p<.005, *=p<.05 and ns = not significant. Means ± s.d. shown.

Mener et al. Figure 4

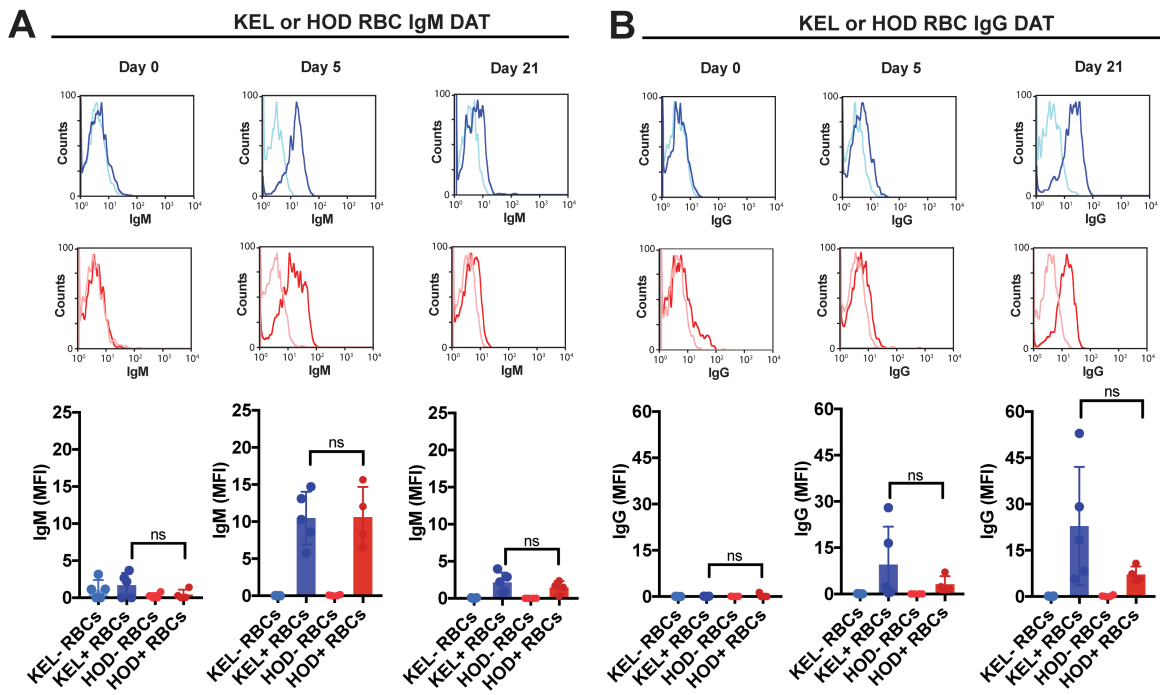


Figure 4: IgM and IgG specifically deposit on KEL or HOD RBCs at similar levels post transfusion. (**A**) KEL negative RBCs (KEL$^-$ RBCs), KEL positive RBCs (KEL+ RBCs), HOD negative RBCs (HOD- RBCs) or HOD positive RBCs (HOD+ RBCs) were evaluated at days 0, 5 and 21 post-transfusion for surface engagement of IgM by flow cytometry in B6 mice. (**B**) KEL negative RBCs (KEL- RBCs), KEL positive RBCs (KEL+ RBCs), HOD negative RBCs (HOD- RBCs) or HOD positive RBCs (HOD+ RBCs) were evaluated at days 0, 5 and 21 post-transfusion for surface engagement of IgG by flow cytometry in B6 mice. For (**A-B**), ns = not significant. Means ± s.d. shown.

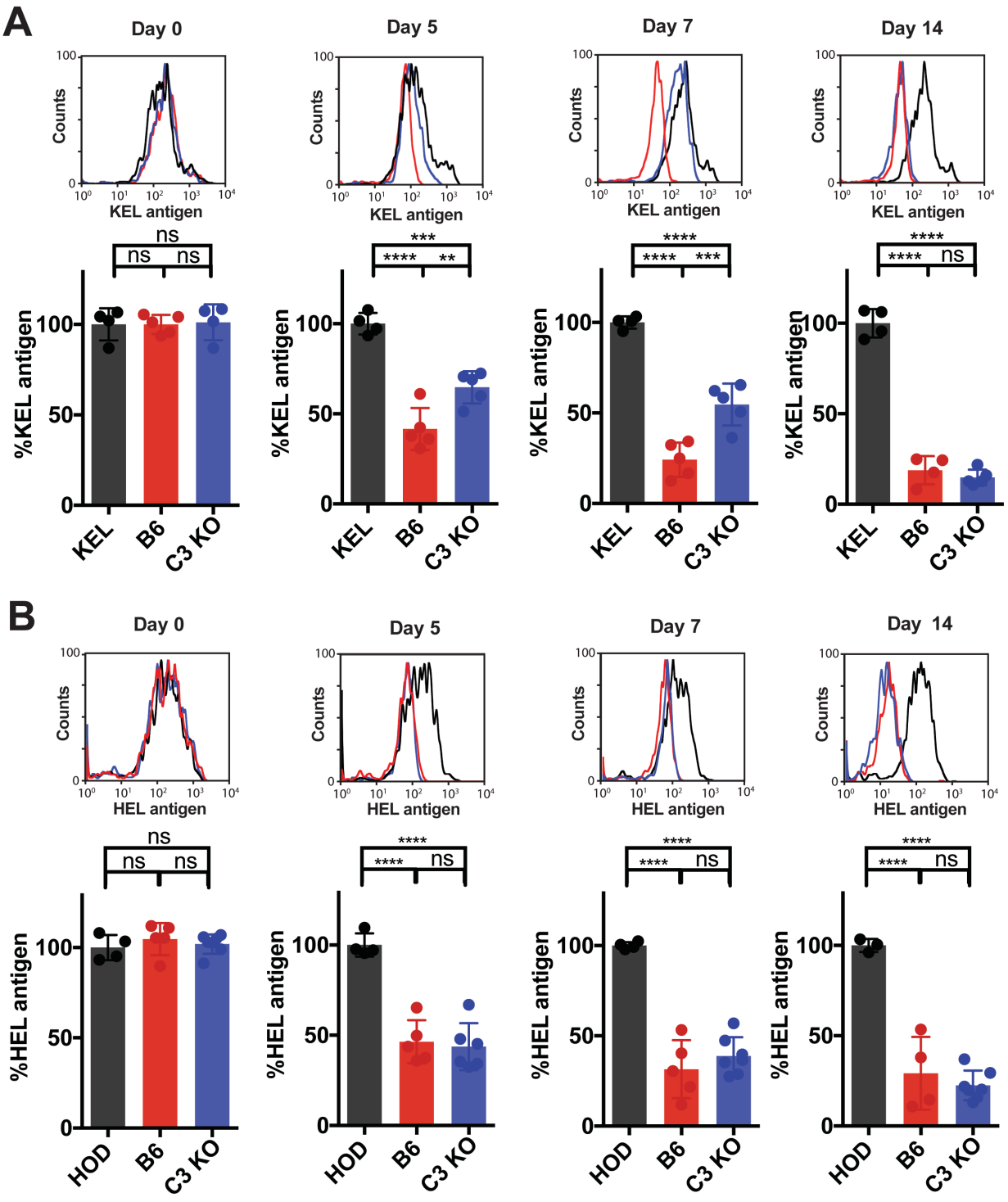


Figure 5: C3 accelerates loss of detectable KEL antigen on KEL RBCs over time.

(**A-B**) B6 or C3 KO recipients were transfused with either DiI-labeled KEL RBCs or DiI-labeled HOD RBCs with control DiO-labeled antigen negative RBCs. As a control, KEL

mice were transfused with syngeneic DiI-labeled KEL RBCs or HOD mice were transfused with syngeneic DiI-labeled HOD RBCs, each with control DiO-labeled antigen negative RBCs. (**A**) Post-transfusion, DiI-labeled KEL RBCs were stained for the level of detectable KEL antigen using anti-KEL polyclonal antibody. Level of detectable KEL antigen was measured as a percentage of KEL antigen normalized to the level of detectable KEL antigen in KEL mice transfused with syngeneic DiI-KEL RBCs, shown at days 0, 5, 7 and 14 post-transfusion. (**B**) Post-transfusion, DiI-labeled HOD RBCs were stained for the level of detectable HEL antigen using polyclonal anti-HEL antibody. Level of detectable HEL antigen was measured as a percentage of HEL antigen normalized to the level of detectable HEL antigen in HOD mice transfused with syngeneic DiI-HOD RBCs. For (**A**-**B**), ****=p<.0001, ***=p<.0008, **=p<.007 and ns = not significant. Means ± s.d. shown.

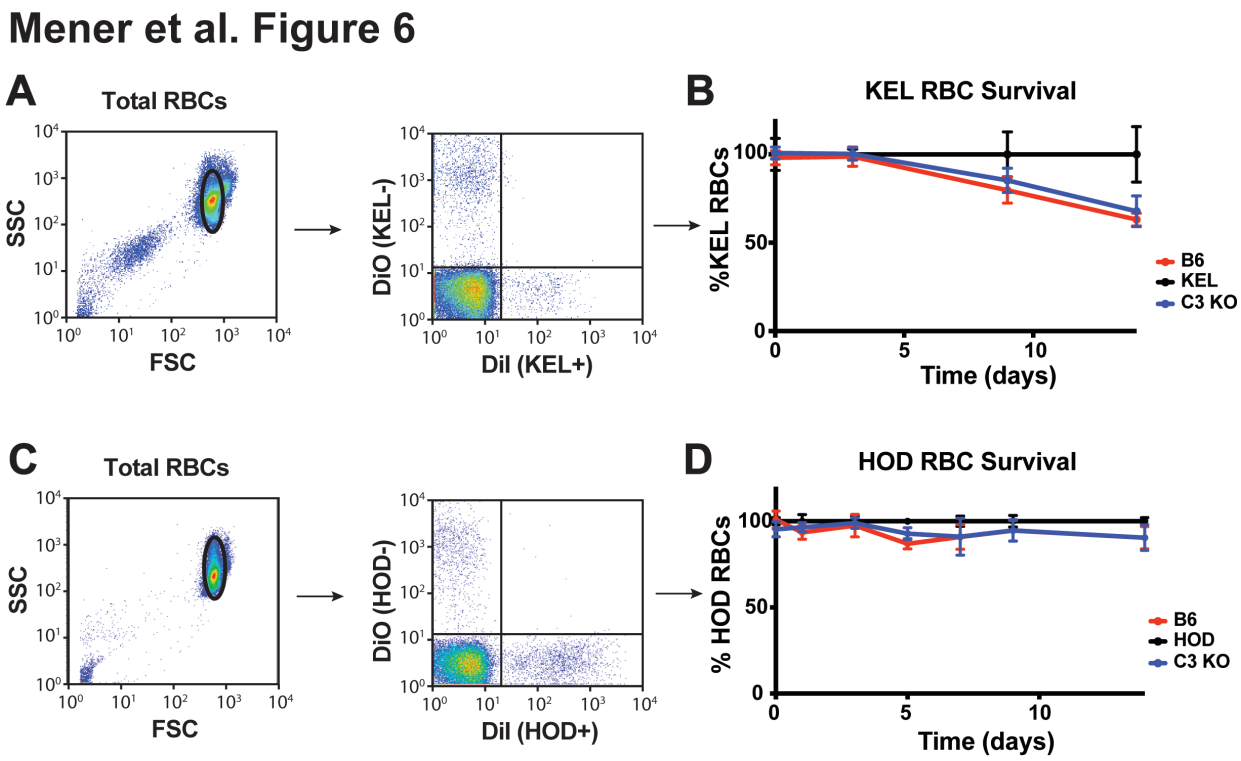


Figure 6: C3 fails to induce detectable alterations in KEL or HOD RBC clearance post-transfusion. (**A**) Gating strategy of KEL+ or KEL- RBCs post-transfusion, where the percentage of KEL+ RBCs was directly compared to KEL- RBCs in each recipient. (**B**) KEL+ RBC survival was calculated as a ratio of KEL+ RBC to KEL- RBC in KEL mice, B6 or C3 KO recipients at the times indicated. (**C**) Gating strategy of HOD+ or HOD- RBCs post-transfusion, where the percentage of HOD+ RBCs was directly compared to HOD- RBCs in each recipient. (**D**) HOD+ RBC survival was calculated as a ratio of HOD+ RBC to HOD- RBC in HOD mice, B6 or C3 KO recipients at the times indicated. For (**B**) and (**D**), RBC clearance was not significant between B6 and C3 KO recipients for KEL RBC (**B**) or HOD RBC (**D**) clearance by Two-way ANOVA with Dunnett's multiple comparisons test. Means ± s.d. shown.

Mener et al. Figure 7

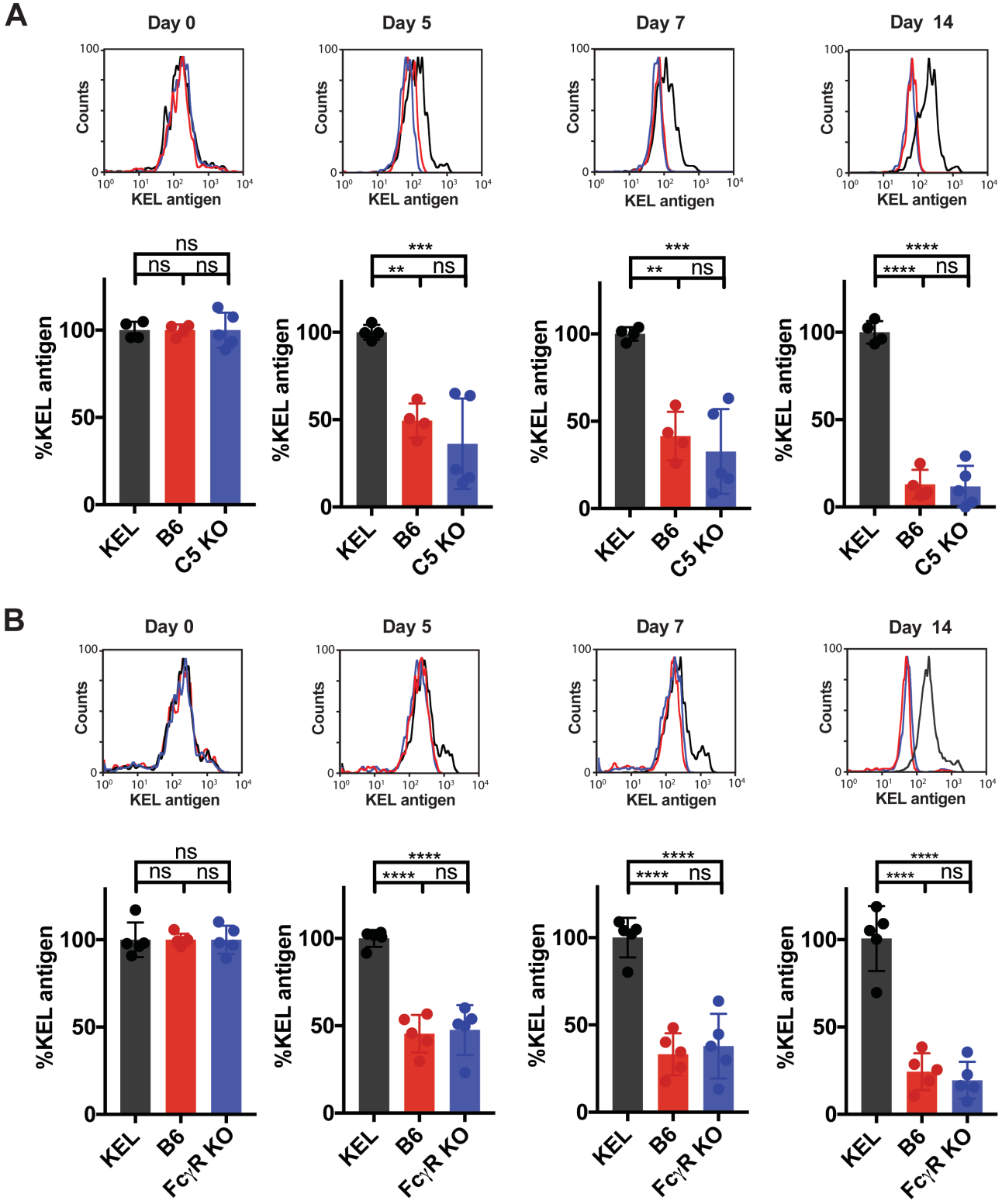


Figure 7: C5 and activating FcγRs do not impact KEL antigen levels on transfused KEL RBCs over time. (**A-B**) B6, C5 KO and FcγR KO mice were transfused with DiI-labeled KEL+ RBCs and DiO-labeled KEL- RBCs. As a control, KEL mice were transfused with syngeneic DiI-labeled KEL+ RBCs and DiO-labeled KEL- RBCs. Post-transfusion, DiI-labeled KEL+ RBCs were stained for level of detectable KEL antigen using an anti-KEL polyclonal antibody. The level of detectable KEL antigen was measured as a percentage of KEL antigen normalized to the level of detectable KEL antigen in KEL mice transfused with syngeneic DiI-KEL+ RBCs, shown at days 0, 5, 7 and 14 post-transfusion for either C5 KO (**A**) or FcγR KO (**B**) mice. For (**A-B**), ****=p<.0001, ***=p<.0007, **=p<.006 and ns = not significant. Means ± s.d. shown.

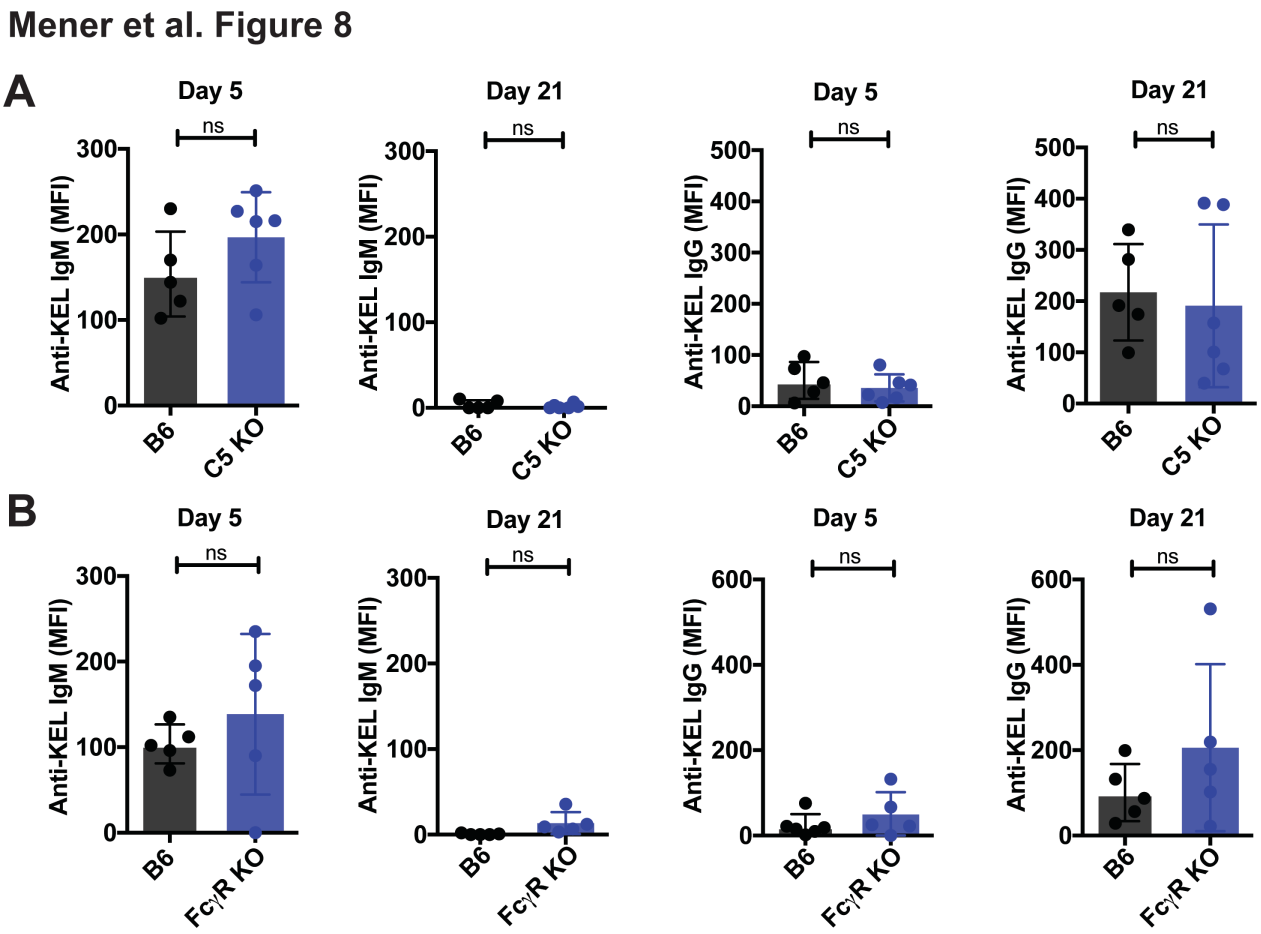


Figure 8: C5 KO and FcγR KO mice exhibit no increase in the anti-KEL antibody response following KEL RBC transfusion. (**A-B**), B6, C5 KO or FcγR KO mice were transfused with KEL RBCs. Anti-KEL IgM and IgG was measured at days 5 and 21 in the serum of C5 KO (**A**) or FcγR KO mice (**B**). For (**A-B**), ns = not significant. Means ± s.d. shown.

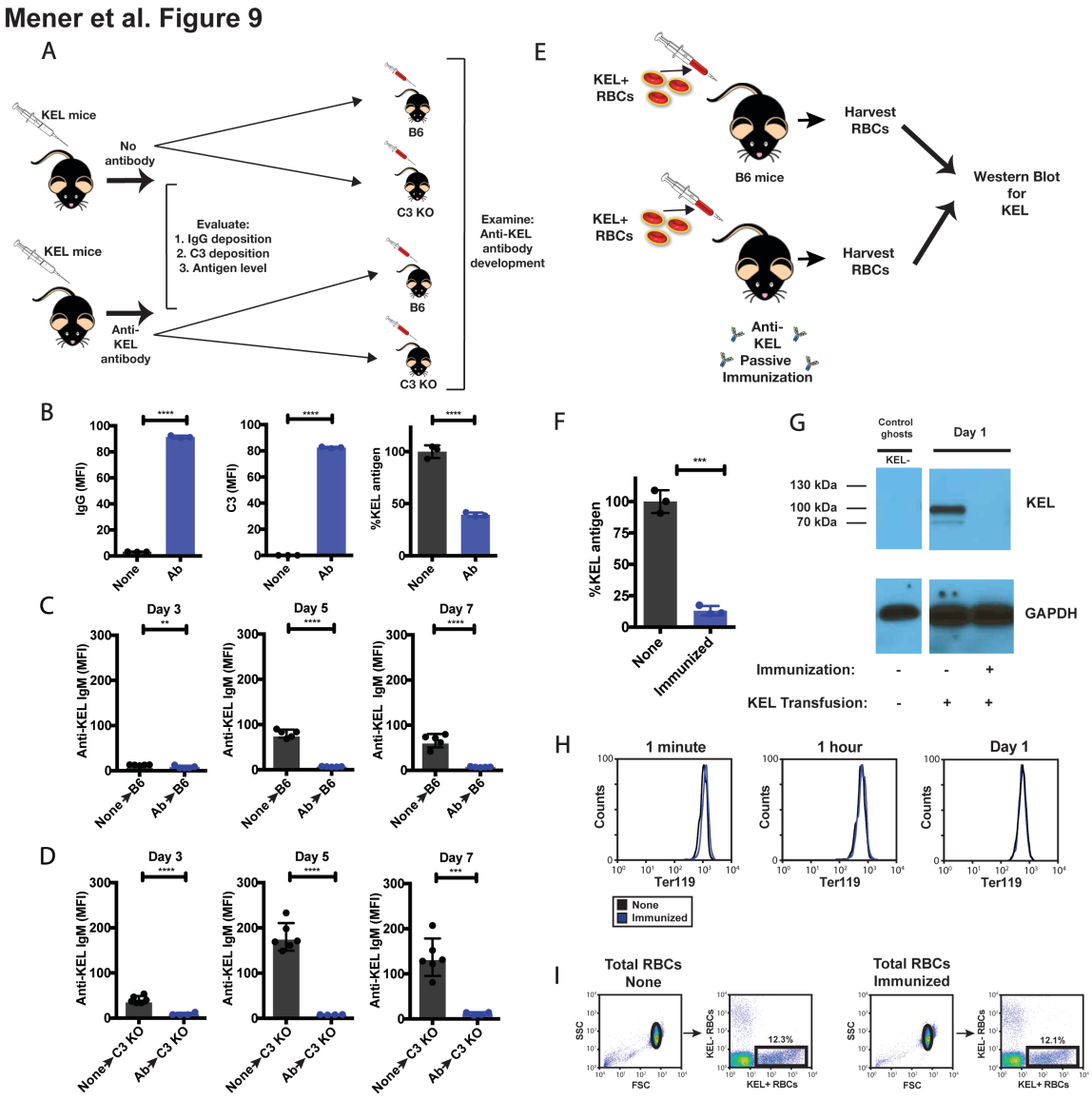
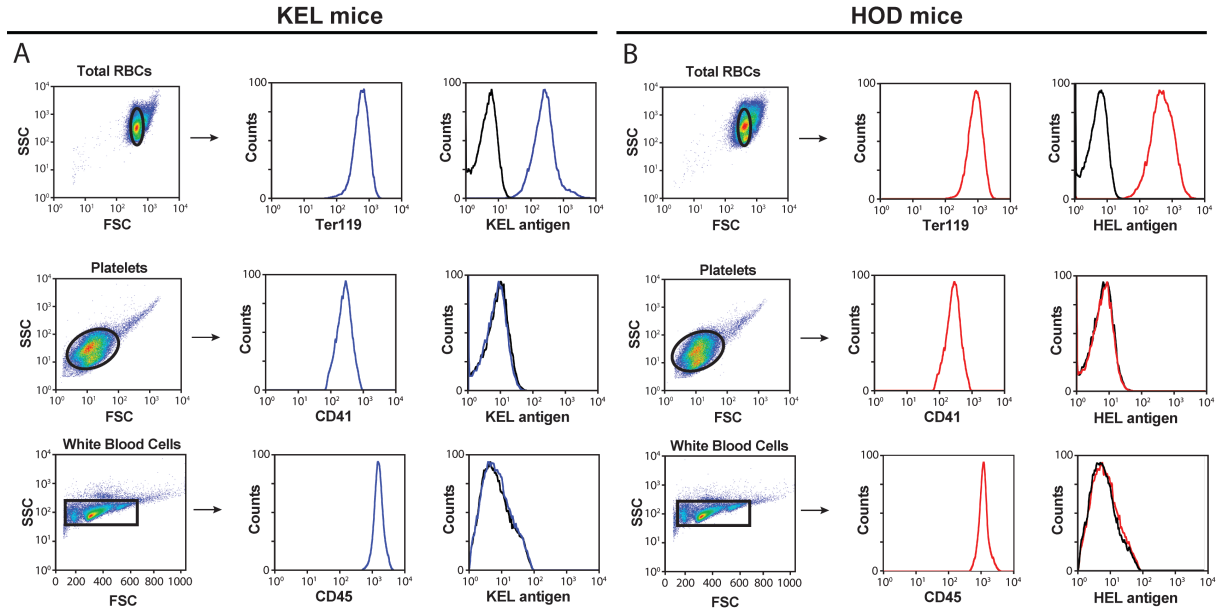


Figure 9: Re-transfusion of anti-KEL polyclonal antibody treated KEL RBCs into B6 or C3 KO mice results in reduced anti-KEL antibody formation. (**A**) Schematic overview: Anti-KEL polyclonal antibody (Ab) or PBS (none) was injected into KEL donors, followed by initial evaluation for IgG deposition, C3 deposition and KEL antigen levels. These isolated RBCs were then retransfused into B6 or C3 KO recipients, which were subsequently evaluated for anti-KEL antibody formation. (**B**) Following injection, IgG, C3 and detectable KEL antigen were assessed on transfused KEL RBCs in KEL

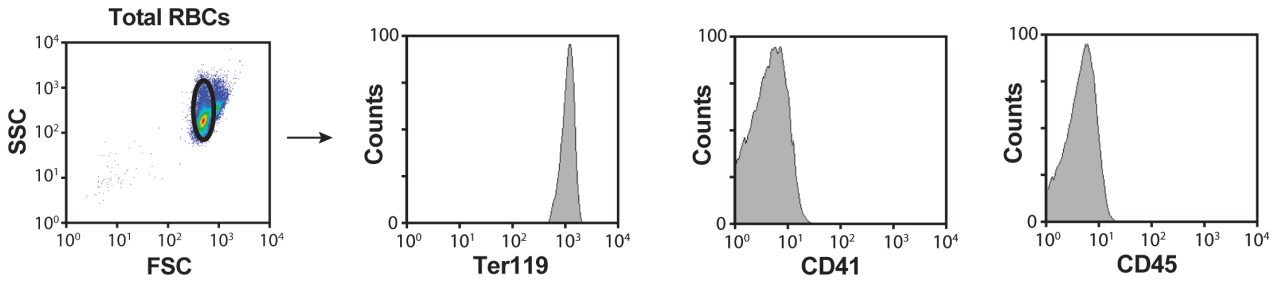
donors. (**C**-**D**), KEL RBCs from PBS (none) or anti-KEL antibody treated (Ab) KEL donors were harvested, followed by transfusion into KEL negative naïve B6 (**C**) or C3 KO recipients (**D**) and assessed for anti-KEL IgM antibodies on days 3, 5, and 7 post-transfusion. (**E**) Schematic overview: KEL negative B6 mice were exposed to KEL RBCs in the absence (None) or presence of polyclonal anti-KEL antibody (Immunized) at a volume adjusted ratio to ensure equivalent KEL RBCs were present in each group 1 day following transfusion. Day 1 post-transfusion, the level of detectable KEL antigen was measured by flow cytometry and western blot analysis. (**F-G**) Day 1 post-transfusion, the level of detectable KEL antigen was measured by flow cytometry **(F)** or western blot analysis **(G)**. (**H**) The level of KEL RBC Ter119 expression was assessed specifically on DiI-labeled transfused KEL positive RBCs at 1 minute, 1 hour and Day 1 post-transfusion in immunized and non-immunized B6 mice by flow cytometry. (**I**) Gating strategy shows KEL RBC population in the absence (None) or presence of polyclonal anti-KEL antibody (Immunized). For (**B**-**F**), **=p<.009, ***=p<.0004 and ****=p<.0001. Means ± s.d. shown.

Mener et al. Supplemental Figure S1

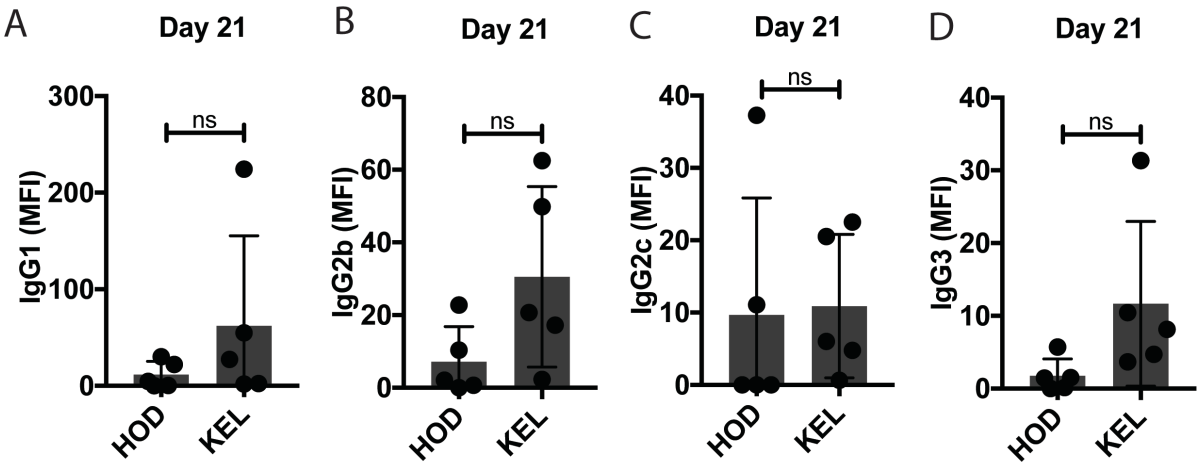


Supplemental Figure S1: KEL or HOD expression is limited to RBCs. (**A**) Red blood cells (RBCs), platelets and white blood cells (WBCs) from KEL donor mice were assessed for KEL expression, along with the lineage-specific markers Ter119, CD41 and CD45, respectively. (**B**) RBCs, platelets and WBCs from HOD donor mice were assessed for HEL expression, along with the lineage-specific markers Ter119, CD41 and CD45, respectively.

Mener et al. Supplemental Figure S2

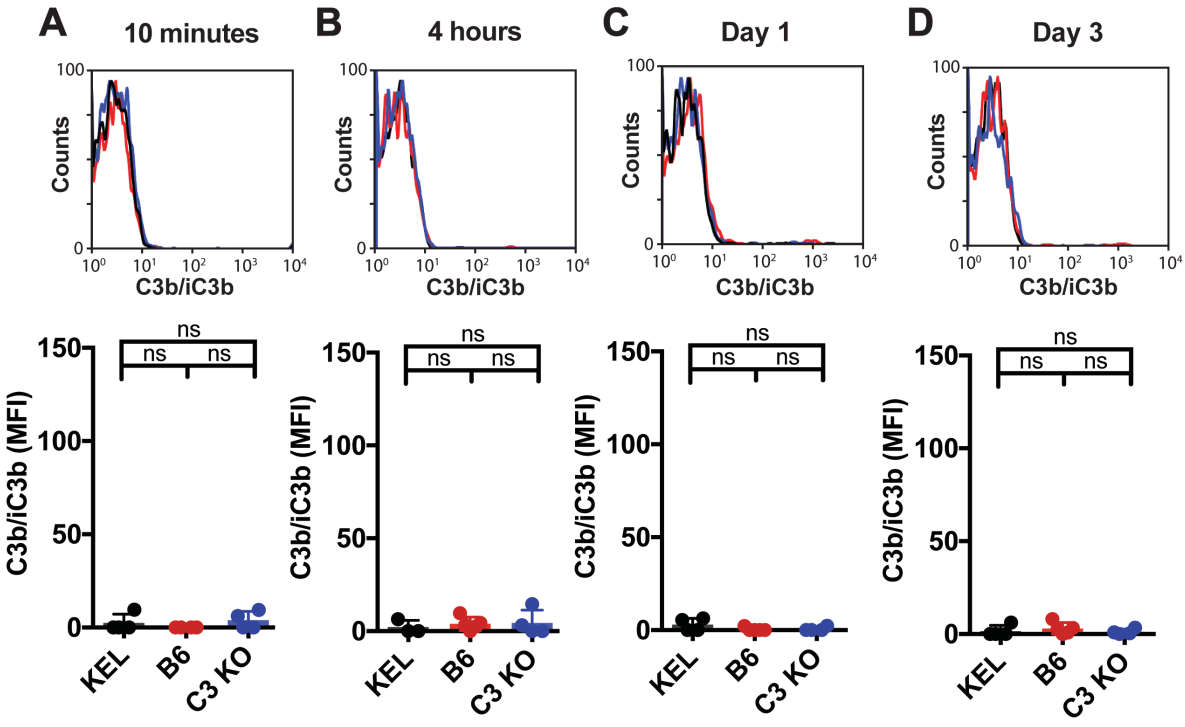


Supplemental Figure S2: Gating strategy for examination of RBCs. Ter119, CD41 and CD45 were assessed specifically on cells within the RBC gate.



Supplemental Figure S3: HOD RBCs and KEL RBCs induce similar anti-HOD and anti-KEL IgG subclass distribution following transfusion. B6 mice were transfused with either HOD or KEL RBCs, followed by evaluation of anti-KEL or anti-HOD IgG1 (**A**), IgG2b (**B**), IgG2c (**C**) and IgG3 (**D**) in the serum on day 21 post-transfusion by flow crossmatch. For A-D, ns = not significant. Means ± s.d. shown.

Mener et al. Supplemental Figure S4



Supplemental Figure S4: Anti-KEL antibodies fail to induce detectable C3b/iC3b deposition on circulating KEL RBCs post-transfusion. C3b/iC3b deposited on circulating DiI-labeled KEL RBCs was measured at 10 minutes (**A**), 4 hours (**B**), day 1 (**C**) and day 3 (**D**) post-transfusion into KEL, B6 and C3 KO mice. For (**A-D**), ns = not significant. Means ± s.d. shown.

Chapter 5: Complement serves as a switch between CD4 T cell independent and dependent RBC antibody responses

Abstract

Red blood cell (RBC) alloimmunization represents a significant immunological challenge for patients requiring life-long transfusion support. The majority of clinically relevant non-ABO(H) blood group antigens have been thought to drive antibody formation through T cell-dependent immune pathways. Thus, we initially sought to define the role of $CD4^+$ T cells in alloantibody formation to KEL, one of the leading causes of hemolytic transfusion reactions. Unexpectedly, our findings demonstrate that KEL RBCs actually possess the ability to induce antibody formation independent of $CD4^+$ T cells or complement component 3 (C3), two common regulators of antibody formation. However, despite the ability of KEL RBCs to induce anti-KEL antibodies in the absence of complement, removal of C3 or complement receptors 1 and 2 (CR1/2) rendered recipients completely reliant on $CD4^+$ T cells for IgG anti-KEL antibody formation. Together, these findings suggest that C3 may serve as a novel molecular switch that regulates the type of immunological pathway engaged following RBC transfusion.

Introduction

Patients with low or dysfunctional red blood cells (RBCs) often require chronic RBC transfusion support to maintain proper tissue oxygenation. This vital therapy substantially diminishes complications in patients with congenital hemoglobinopathies, including sickle cell anemia and β-thalassemia. However, chronic RBC transfusion support is not without risk. Repeat exposure to antigenic variations between donor and recipient can lead to an undesirable immunological barrier to transfusion, with up to 20-50% of chronically transfused patients developing antibodies against allogeneically distinct RBC antigens (1-3). Formation of alloantibodies compromises the therapeutic efficacy of transfused cells and reduces the availability of compatible RBCs for future transfusions (1, 4-6). Alloantibodies against RBC antigens can also cause hemolytic disease of the fetus and newborn (7, 8) and increase the probability of hemolytic transfusion reactions, one of the leading causes of transfusion related mortality (2). Ultimately, RBC alloimmunization directly increases the morbidity and mortality of transfusion dependent patients (6, 9). While antigen-matching protocols can reduce the probability of RBC alloimmunization and prophylactic use of anti-D globulin can prevent anti-D antibody formation, neither of these approaches completely prevents RBC alloimmunization (10). Unfortunately, no therapeutic modality currently exists that actively prevents the rate of humoral immunization to a RBC alloantigen (2, 10-12). As a result, understanding the mechanism(s) by which RBC alloantibodies develop may aid in the identification of key targets that can be used to inhibit RBC alloimmunization in chronically transfused individuals.

Excluding the well-established ABO(H), I and other carbohydrate RBC antigens, the vast majority of clinically relevant blood group antigens (i.e. Kell, Kidd, Duffy, etc) are proteins or glycoproteins that are thought to lack fundamental biochemical properties of T cell independent antigens (13). Rather, these non-carbohydrate blood group antigens have been uniformly thought to induce antibody responses through $CD4^+$ T cell help. Consistent with this, certain HLA Class II alleles correlate with the risk of developing alloantibodies against some RBC antigens (14-23). This has led to $CD4^+$ T cells becoming the primary focus of possible strategies designed to inhibit RBC alloimmunization in chronically transfused individuals (24-28). We recently demonstrated in a murine model of RBC alloimmunization that antigen specific $CD4^+$ T cells are indeed required for the development of alloantibodies to the model RBC antigen, HOD, a trimeric fusion protein consisting of hen egg lysozyme, ovalbumin, and human blood group antigen Duffy (24). Previous correlative clinical studies together with these recent findings in the HOD animal model support the hypothesis that T-B cell cooperation is vital for the formation of alloantibodies to RBC antigens. However, every RBC alloantigen is very distinct and differs in structure and overall function, suggesting that while various RBC antigens can induce alloantibody responses, the immune pathways they engage may fundamentally differ.

Given the apparent role of $CD4^+$ T cells in RBC alloantibody formation, we initially sought to characterize the role $CD4^+$ T cells play in orchestrating formation of alloantibodies against KEL, one of the most common alloantigens implicated in hemolytic transfusion reactions and hemolytic disease of the fetus and newborn (8, 29-33). As mechanistic studies in humans are not justifiable and mice do not inherently

express RBC polymorphisms capable of inducing an alloantibody response following RBC exposure, we generated a mouse model of RBC alloimmunization by expressing the human KEL antigen specifically on RBCs using a β-globin promoter (7, 34-37). Using this model system, we unexpectedly found that depletion or genetic deletion of $CD4^+$ T cells failed to impact KEL reactive alloantibody formation. Furthermore, though KEL reactive alloantibodies generated in the presence or absence of $CD4^+$ T cells fixed complement component 3 (C3), similar to many clinically relevant alloantibodies (38-41), alloantibody formation to KEL in the absence of C3 or complement receptors 1 and 2 (CR1/2) was found to be dependent on $CD4^+$ T cells. Overall, these data demonstrate that C3–CR1/2 ligation has the potential to dictate whether an antibody response to a distinct RBC protein antigen induces antibody formation independent or dependent of $CD4^+$ T cell help. These results illustrate that different RBC alloantigens may therefore possess the ability to engage distinct immune pathways depending on complement fixation following transfusion.

Materials and Methods

Mice

Female B6 (CD45.2 C57BL/6; H-2^b) and CD45.1 B6 (B6.SJL-PtprcaPepcb/BoyCrCrl) mice were purchased from the National Cancer Institute (Frederick, MD) or Charles River (Wilmington, MA). μMT (B6.129S2-*Ighm*^{tm1Cgn}/J; H-2^b), MHC Class II KO (B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J; H-2^b), TCRα KO (B6.129S2-*Tcra*^{tm1Mon}/J; H-2^b), and C3 KO mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6 – 8 weeks of age. Genetic deletion of C3 in C3 KO mice was confirmed by isolating DNA from peripheral blood using the DNeasy Blood and Tissue Kit (QIAGEN) and performing a polymerase chain reaction using Taq Polymerase 2x Master Mix Red (Apex). The primer sequences utilized for genotyping C3 KO mice were provided by The Jackson Laboratory: a common primer olMR1325 (sequence: ATC TTG AGT GCA CCA AGC C), a wild-type primer olMR1326 (sequence: GGT TGC AGC AGT CTA TGA AGG) and a mutant primer olMR7415 (sequence: GCC AGA GGC CAC TTG TGT AG). CR1/2 deficient mice (CR1/2 KO) were a generous gift from V. Michael Holers, MD at the University of Colorado Denver (Denver, CO). All mice were on a B6 background and used at 8-12 weeks of age. Transgenic KEL (H-2^b) donors were a generous gift from Dr. James C. Zimring [Puget Blood Institute, Seattle, WA (34)]. Mice were bred and housed in Emory University Department of Animal Resources facilities, and all procedures were performed according to approved Institutional Care and Use Committee (IACUC) protocols.

Antibodies for Flow Cytometry

APC rat anti-mouse CD4, FITC rat anti-mouse CD3ε, PE rat anti-mouse CD8α, APC Rat IgG2a, κ, FITC rat anti-mouse CD45R/B220, PE CF594 rat anti-mouse CD45R/B220, APC rat anti-mouse CD21/CD35, PE rat anti-mouse CD23, PE anti-mouse CD1d, APC streptavidin, PE anti-mouse CD45.1 + PerCP-Cy5.5 anti-mouse CD45.2, Brilliant Violet 785 anti-mouse CD3, V500 anti-mouse CD4, PE Cy7 anti-mouse CD8, V450 anti-mouse B220, APC Cy7 anti-mouse CD21/35, PE Texas Red anti-mouse CD11b, Brilliant Violet 605 anti-mouse CD11c, Alexa Fluor 488 anti-mouse NK1.1, and Alexa Fluor 647 anti-mouse Ly6C were bought from BD bioscience (San Jose, CA). Biotinylated rat anti-mouse C3 was obtained from Cedarlane (Burlington, Canada), and APC goat anti-mouse IgG, FITC goat anti-mouse IgM, PE goat anti-mouse IgG1, PE goat anti-mouse IgG2c, PerCP goat anti-mouse IgG3, Alexa Flour 488 goat anti-mouse IgG2b and anti-mouse IgG HRP were purchased from Jackson Immunoresearch (West Grove, PA).

Cellular depletion and RBC transfusion

$CD4^+$ T cell depletion was achieved by two intra-peritoneal injections of 250 μg monoclonal anti-mouse CD4 depleting antibody (clone: GK1.5; Bio X Cell, West Lebanon, NH) diluted in 1x DPBS, 4 and 2 days prior to transfusion (42). Efficacy of $CD4^+$ T cell depletion was assessed by staining peripheral blood (prior to transfusion) or splenocytes with FITC rat anti-mouse CD3ε + PE rat anti-mouse CD8α + APC rat anti-mouse CD4 (clone: RM-45). Percent $CD4^+$ T cells were computed by gating on $CD3^+$ $CD4^+$ $CD8^-$ T cells. Samples were run on a 4 color BD FACSCalibur and analyzed using FlowJo. Donor KEL whole blood was collected 1:8 into acid citrate dextrose (ACD,

Vacutainer, Franklin Lakes, NJ) and washed 3 times with 1x DPBS. Depleted recipients were then transfused via the lateral tail vein with 50 μl of packed KEL RBCs diluted in 1x DPBS to 300 μl total volume (equivalent to 1 human unit).

LCMV infection and anti-LCMV IgG ELISA

B6 recipients depleted of $CD4^+$ T cells were infected with LCMV and assayed for anti-LCMV antibody formation by ELISA, as previously described (43-47). Briefly, B6 recipients treated with PBS or a monoclonal anti-mouse CD4 depleting antibody (clone: GK1.5) two times a day apart were infected with 2×10^6 plaque forming units (PFU) of LCMV (clone 13). Serum was then collected 15 days post-infection and tested for anti-LCMV IgG formation through ELISA. Serial dilutions of serum were added to MaxiSorp plates (Nunc) previously coated overnight with LCMV lysate or an anti-mouse IgG antibody. Bound anti-LCMV IgG was then detected using a secondary anti-mouse IgG HRP antibody diluted 1:5000 in blocking buffer.

Survival of RBCs and C3 fixation on transfused RBCs in vivo

Wild type B6 or transgenic KEL whole blood was collected 1:8 into ACD and washed three times with 1x DPBS. Resulting B6 packed RBCs were labeled with 3, 39-dihexadecyloxacarbocyanine perchlorate (DiO; Molecular Probes, Eugene, OR), while KEL packed RBCs were labeled with chloromethylbenzamido 1, 19-dioctadecyl-3, 3, 39, 39-tetramethylindocarbocyanine perchlorate (CM-DiI; Molecular Probes, Eugene, OR), as previously described (37). Briefly, 1 mL packed RBCs were diluted 1:10 in 1x DPBS. DiO or DiI was next added to the respective RBC samples at a 1:100 dilution. Samples

were incubated for 30 minutes at 37°C and then washed three times to remove any unbound dye. Both populations were subsequently mixed at a 1:1 ratio and recipients were transfused via lateral tail vein with 50 µl of each type of blood diluted in 1x DPBS to a total volume of 300 µl. At day 0 or 3, 5, 7 and 14 days post transfusion, KEL RBC survival and C3 fixation were examined. Survival of transfused KEL-DiI RBCs was measured by normalizing percent KEL-DiI RBCs to tracer B6-DiO RBCs. C3 fixation was assessed by incubating samples for 30 minutes at 4°C with biotinylated anti-mouse C3 antibody diluted 1:100 in 1x DPBS + 2% bovine serum albumin (BSA) buffer, followed by APC streptavidin diluted 1:100 in 1x DPBS + 2% BSA buffer for 30 minutes at 4°C. All samples were run on a 4-color BD FACSCalibur and analyzed by FlowJo; mean fluorescence intensity was used to measure C3 fixation on transfused KEL-DiI RBCs.

Seroanalysis

Serum collected 5, 14 and 28 days post transfusion was evaluated for anti-KEL antibodies by indirect immunofluorescence staining, as previously described (37). Briefly, neat serum or serum at indicated titrations (diluted in PBS) was incubated with packed KEL or B6 RBCs for 15 minutes at room temperature. After 15 minutes, samples were washed three times with FACS buffer (1x DPBS + 2% bovine serum albumin + 0.9 g EDTA), and incubated for 30 minutes in a 1:100 dilution of an antibody cocktail consisting of APC anti-mouse IgG, FITC anti-mouse IgM, and PE anti-mouse PE or Alexa 488 anti-mouse IgG2b, PE anti-mouse IgG2c, and PerCP anti-mouse IgG3. Samples were run on a 4-color BD FACSCalibur and analyzed using FlowJo; mean

fluorescence intensity of indicated fluorophores was used to measure the amount of antigen specific antibody subsets present in the serum. Indeed, anti-KEL antibodies are technically not "alloantibodies". However, previous work using the KEL RBC model system has commonly referred to anti-KEL antibodies as "alloantibodies" (7, 35, 37, 48). Thus, to continue uniformity of nomenclature within the field, we have continued to use "alloantibodies" to describe antibodies generated in response to KEL on transfused KEL RBCs.

Bone Marrow Transplantation

CD45.1 B6 and CR1/2 KO recipients were irradiated with two doses of 550 Gy three hours apart using a gamma irradiator. 24 hours later, bone marrow was harvested from CD45.2 B6, CD45.1 B6, and/or CR1/2 KO donors, as previously described (49). Briefly, marrow was flushed from the femurs with 5% FBS in RPMI using a 25G needle. Marrow was then passed through an 18G needle to homogenize the marrow. The marrow was then filtered through a 70 μm filter to remove debris. Marrow was then centrifuged for 10 minutes at 1200 rpm and washed two times with 1x PBS. Cells were counted using a hemocytometer and re-constituted in 1x PBS to 10 x 10^6 cells/mL. 500 μL of the bone marrow was then transfused into indicated recipients via the lateral tail vein (5 x 10^6 bone marrow cells total). Post-transplantation, recipients were treated with 1 mg/mL neomycin sulfate (Sigma) in sterile drinking water that was changed weekly. Reconstitution was determined 10 weeks post-transplantation by staining peripheral blood leukocytes with PE anti-mouse CD45.1 + PerCP-Cy5.5 anti-mouse CD45.2, Brilliant Violet 785 anti-mouse CD3, V500 anti-mouse CD4, PE Cy7 anti-mouse CD8,

V450 anti-mouse B220, APC Cy7 anti-mouse CD21/35, PE Texas Red anti-mouse CD11b, Brilliant Violet 605 anti-mouse CD11c, Alexa Fluor 488 anti-mouse NK1.1, and Alexa Fluor 647 anti-mouse Ly6C. 10^6 total leukocytes were collected and FlowJo was used to determine percent engraftment of distinct leukocyte populations. Following engraftment, mice were depleted of $CD4^+$ T cells 4 and 2 days prior to transfusion. All recipients were transfused with 50 μL packed KEL RBCs in 300 μL of 1x PBS by lateral tail vein injection.

Statistics

Statistical analysis was performed using one-way ANOVA with Dunnett's or Tukey's post-test, or student t-test. Significance was determined by a P value less than 0.05.

Results

KEL alloantibody formation occurs independent of CD4$^+$ T cell help

While several previous studies examining the immune response to KEL suggest that distinct levels of complement deposition and CD4$^+$ T cell help may occur following KEL exposure (7, 34-37, 50, 51), we first sought to formally test the hypothesis that cognate T-B cell interactions are required for alloimmunization to KEL on transfused RBCs. To do this, wild type B6 and MHC Class II knockout (MHC II KO) recipients that are genetically deleted of MHC Class II molecules and consequently deficient in CD4$^+$ T cells (Figure 1A) were transfused with KEL RBCs. Recipients were transfused with a volume-adjusted equivalent of 1 human unit of KEL RBCs (7, 35-37, 52, 53). Serum was then collected and evaluated for the presence of anti-KEL IgM and IgG alloantibodies through indirect immunofluorescence staining using KEL or B6 RBCs (Supplementary Figure 1A-B), a previously established method used clinically and experimentally to evaluate anti-RBC antibody formation (24, 35-37).

Surprisingly, transfusion of KEL RBCs into MHC II KO recipients generated an anti-KEL IgM and IgG response that was not statistically different than the antibody response observed in KEL RBC transfused B6 wild type recipients (Figure 1B). While indirect immunofluorescence staining represents the gold standard for examining antibody production in the transfusion medicine field, it is possible that the potential impact CD4$^+$ T cell deficiency could have had on the anti-KEL antibody response may not have been detected using this approach. Thus, to determine whether differences in antibody titer were present in MHC II KO recipients transfused with KEL RBCs, anti-KEL antibody formation was evaluated using indirect immunofluorescence staining over

serial serum titrations. Similar to the anti-KEL antibody response observed using neat serum, serum titration demonstrated no statistically significant difference in total anti-KEL IgG in MHC II KO and wild type B6 recipients transfused with KEL RBCs (Supplementary Figure 1C). However, $CD4^+$ T cell deletion is not complete in MHC II KO recipients (Figure 1A) (54). Thus, to additionally examine the role of $CD4^+$ T cell help in KEL alloimmunization, B6 recipients negative for KEL were administered PBS (B6) or a CD4 depleting antibody (clone: GK1.5) two times, a day apart. $CD4^+$ T cell depletion efficacy was assessed in the spleen of representative recipients to assure $CD4^+$ T cell depletion prior to transfusion of KEL RBCs (Figure 1C).

Analogous to MHC II KO recipients, transfusion of KEL RBCs into recipients depleted of $CD4^+$ T cells generated an anti-KEL IgM and IgG response that was not statistically different than the antibody response in KEL RBC transfused PBS (B6) treated recipients (Figure 1D and Supplementary Figure 1D). The inability to block an alloantibody response to KEL in $CD4^+$ T cell depleted recipients was not likely due to insufficient depletion, as B6 recipients depleted of $CD4^+$ T cells in parallel failed to generate anti-LCMV antibodies (Supplementary Figure 2A), and $CD4^+$ T cells were undetectable in the peripheral blood immediately prior to transfusion (Supplementary Figure 2B) and in the spleen of representative recipients evaluated in parallel (Figure 1C). As an additional measure to confirm whether KEL RBCs can induce anti-KEL antibody formation in the absence of $CD4^+$ T cells, T cell receptor α (TCRα) KO recipients that are genetically deficient of $CD4^+$ T cells were transfused with KEL RBCs (55) (Supplementary Figure 3A). Similar to MHC II KO and CD4 depleted recipients, there was no statistically significant difference in anti-KEL IgG in TCRα KO and wild type B6

recipients following KEL RBC transfusion (Supplementary Figure 3B). Together, these findings demonstrate that MHC dependent $CD4^+$ T cell help is not required for the generation of a humoral immune response to KEL.

$CD4^+$ T cell help does not play a role in polarization of a humoral response to KEL

Although direct $CD4^+$ T cell help was found to be inessential for the formation of KEL reactive alloantibodies, it is plausible that MHC independent $CD4^+$ T cell help may be necessary to qualitatively, and thereby functionally shape the humoral response to KEL. In particular, there is significant evidence demonstrating the immunological influence $CD4^+$ T cell derived cytokines have on IgG subclass switching following B cell recognition of both T cell dependent and T cell independent antigens (56). Accordingly, we tested the hypothesis that though not required to produce anti-KEL IgG, $CD4^+$ T cells may dictate the type of IgG alloantibodies produced in response to a KEL RBC transfusion. To accomplish this, sera from recipients depleted or genetically deleted of $CD4^+$ T cells was additionally evaluated for the presence of anti-KEL IgG1, IgG2b, IgG2c, and IgG3 alloantibodies. Transfusion of KEL RBCs into MHC II KO recipients produced comparable levels of anti-KEL IgG1, IgG2b, IgG2c, and IgG3 levels that were statistically similar to PBS (B6) recipients (Figure 2A). Likewise, recipients depleted of $CD4^+$ T cells produced comparable levels of anti-KEL IgG1, IgG2b, IgG2c, and IgG3 levels that were statistically similar to PBS (B6) and isotype control treated recipients (Figure 2B). Similar levels of anti-KEL IgG1, IgG2b, IgG2c, and IgG3 were also observed in KEL RBC transfused TCRα KO recipients compared to wild type B6 recipients (Supplementary Figure 3C).

Alloantibodies generated in the absence of CD4$^+$ T cells fix C3 and facilitate KEL RBC clearance

Despite production of a similar anti-KEL alloantibody response, we next sought to determine whether the KEL reactive alloantibodies generated in the absence of CD4$^+$ T cell help were functionally similar to anti-KEL alloantibodies formed in the presence of CD4$^+$ T cells. To do this, recipients depleted or genetically deleted of CD4$^+$ T cells were transfused with KEL RBCs labeled with a lipophilic dye, DiI, while B6 RBCs were labeled with a fluorescently distinct dye, DiO, to facilitate direct examination of specific changes to KEL RBC survival and complement deposition post-transfusion (Figure 3A). This was accomplished by evaluating the ratio of DiI positive KEL RBCs to DiO positive B6 RBCs, which allows analysis of KEL RBC survival as a function of B6 RBC survival to control for antibody-independent RBC clearance that may reflect potential differences in injection volume, sample collection, and normal cell turnover over time (Figure 3A). Likewise, KEL positive recipients transfused with the RBC mixture were additionally included to control for background C3 fixation and non-immune mediated clearance of KEL RBCs, as these recipients do not generate anti-KEL antibodies (36).

KEL RBC-induced alloantibodies generated in the presence or absence of CD4$^+$ T cell help appeared to be functionally equivalent. Similar to PBS treated B6 recipients (B6), C3 fixation was observed in CD4$^+$ T cell depleted and genetically deleted MHC II KO recipients (Figure 3B-C). Likewise, anti-KEL alloantibodies formed in the absence of CD4$^+$ T cell help correlated with a statistically similar KEL RBC clearance when compared to PBS (B6) treated B6 recipients (Figure 3D). The observed C3 fixation and

RBC clearance was not due to non-specific effects of transfusing labeled KEL RBCs, as C3 fixation and KEL RBC clearance was not observed in KEL positive recipients (Figure 3B-D). Combined, these findings demonstrate that the blood group antigen KEL possesses the inherent capacity to mediate a functional alloantibody response independent of $CD4^+$ T cell help in this model system.

Recipients deficient in C3 mount an enhanced anti-KEL antibody response

Given that C3 deposition was detectable on transfused KEL RBCs (Figure 3B-C) and significant evidence suggests a key role for complement in the development of humoral immunity (57-61), we next tested whether IgG anti-KEL alloantibody formation was dependent on C3. To do this, B6 and C3 deficient (C3 KO) recipients were transfused with KEL RBCs and subsequently evaluated for the production of KEL reactive alloantibodies. Unexpectedly, the absence of C3 not only failed to inhibit the development of anti-KEL antibodies following KEL RBC exposure (Figure 4A-B), but the absence of C3 actually resulted in a statistically significant enhancement of the anti-KEL antibody response (Figure 4A-B). Furthermore, C3 KO recipients transfused with KEL RBCs demonstrated a statistically significant increase in anti-KEL IgG1 and IgG3 production (Figure 4C). These results indicate that while C3 may not be required to induce an antibody response to KEL, the presence of C3 may regulate not only the type but also the magnitude of the immune response induced following KEL RBC transfusion.

Alloantibody formation to KEL is dependent on $CD4^+$ T cells in recipients deficient in C3 or CR1/2

The ability of KEL RBCs to induce an enhanced anti-KEL antibody response stands in stark contrast to previous studies that demonstrate that optimal IgG formation often requires C3 (62, 63). However, given the enhanced and differentially polarized antibody response to KEL observed in the absence of C3 (Figure 4), these results suggest that complement may directly influence the type of immune pathway engaged following KEL RBC exposure. This is especially important when considering that while $CD4^+$ T cells may not be required for anti-KEL antibody formation in the presence of C3 (Figure 1), as $CD4^+$ T cell-derived cytokines can impact the polarization of a IgG subclass response (64-69), it is possible that in the absence of C3, $CD4^+$ T cells may impact anti-KEL alloantibody formation. To test this, wild type B6 and C3 KO recipients were depleted of $CD4^+$ T cells prior to a KEL RBC transfusion and subsequently examined for the development of anti-KEL antibodies. Consistent with the IgM response observed in B6 recipients treated with PBS or CD4 depleting antibody (Figure 1), C3 KO recipients transfused with KEL RBCs developed a comparable anti-KEL IgM response in the presence or absence of $CD4^+$ T cells (Figure 5A). However, in contrast to wild type B6 recipients depleted of $CD4^+$ T cells, $CD4^+$ T cell depleted C3 KO recipients completely failed to generate a detectable anti-KEL IgG response (Figure 5A). As activation of the complement cascade can lead to the downstream generation of C5 (70) and C5 has been shown to play a significant role in $CD4^+$ T cell activation, co-stimulation as well as survival (71, 72), whether C5 is important in the development of a $CD4^+$ T cell independent anti-KEL antibody response was next investigated. However, unlike in the absence of C3, exposure of C5 KO recipients to KEL RBCs in the absence of $CD4^+$ T cells resulted in equivalent anti-KEL antibody formation (Supplementary Figure 4).

These results together suggest that C3 fixation on transfused KEL RBCs may drive the formation of anti-KEL IgG independent of $CD4^+$ T cells and that C3 may therefore represent a novel molecular switch that dictates $CD4^+$ T cell dependent or independent anti-KEL IgG formation following KEL RBC transfusion.

Given that complement receptor ligation with CD19 and the B cell receptor can act as a co-stimulatory signal for B cells (73-76) and C3 KO recipients were unable to mount a detectable anti-KEL IgG response in the absence of $CD4^+$ T cells (Figure 5A), it is possible that when present C3 ligation of CR1/2 may play a critical role in the development of anti-KEL IgG in the absence of $CD4^+$ T cells. Thus, to initially determine whether CR1/2 was important for the development of anti-KEL antibodies, wild type B6 and CR1/2 KO recipients were transfused with KEL RBCs and subsequently tested for the development of antibodies against KEL. Exposure of CR1/2 KO recipients to KEL RBCs resulted in a delayed, but similar anti-KEL IgM response to B6 wild type recipients (Supplementary Figure 5A). However, consistent with the critical role of CR1/2 in B cell responses in general (60, 77), CR1/2 KO recipients transfused with KEL RBCs resulted in a depressed anti-KEL IgG response compared to wild type B6 recipients (Supplementary Figure 5B). Taken together, these results suggest that similar to the immune response to other antigens (58-60, 77-82), CR1/2 expression is important in facilitating the development of anti-KEL IgG antibodies.

As numerous studies examining a variety of different immunogens certainly demonstrate that CR1/2 is an important component of the B cell receptor signaling in general (59, 73, 82-87), it is not surprising that despite the presence of C3 the antibody response to KEL was diminished in recipients deficient in CR1/2 (Supplementary Figure

5). However, given that the T cell independent antibody response to KEL requires C3 (Figure 5A), it remained possible that C3 engagement of CR1/2 possesses the ability to drive IgG formation through a $CD4^+$ T cell independent pathway, and therefore serves as a regulator through which complement dictates whether antibody will form through a T cell independent or dependent pathway. Thus, to investigate whether CR1/2 is necessary to mount a T cell independent anti-KEL IgG response, CR1/2 KO recipients were depleted of $CD4^+$ T cells prior to a KEL RBC transfusion and evaluated for the generation of anti-KEL alloantibodies. KEL RBC transfused CR1/2 KO recipients mounted a detectable anti-KEL IgM response in both the presence and absence of $CD4^+$ T cells (Figure 5B). However, in the absence of $CD4^+$ T cells, CR1/2 KO recipients failed to generate a detectable anti-KEL IgG response compared to PBS treated CR1/2 KO recipients (Figure 5B). Combined, these results suggest that CR1/2 ligation of C3 fixed on transfused KEL RBCs may facilitate a T cell independent antibody response to the KEL antigen. However, in the absence of either C3 or CR1/2, the ability to generate an anti-KEL IgG response becomes dependent on $CD4^+$ T cells. Moreover, these data indicate that the innate immune factor C3 has the potential to serve as a molecular switch capable of regulating whether a humoral immune response to transfused KEL RBCs occurs through a T cell dependent or independent process.

Complement receptor expression on hematopoietic cells determines the requirement of $CD4^+$ T cell help for the formation of an anti-KEL antibody response

Both hematopoietic and non-hematopoietic constituents express CR1/2 and upon ligation of C3 can lead to multiple downstream functions, including opsonization of

complement-coated cells (88-90), formation of the membrane attack complex (91, 92), and facilitation of B cell receptor engagement and antigen presentation (78, 93). However, because complement is mainly synthesized in the liver, along with other extra-hepatic sources, and circulates in the serum (63, 94, 95), it is difficult to elucidate the exact source of C3 that may be responsible for inducing a T cell independent anti-KEL antibody response. Thus, as our data indicate that CR1/2 ligation may also be important in the development of a T cell independent antibody response to transfused KEL RBCs in wild type B6 recipients, and previous studies demonstrate that CR1/2 is only expressed by B cells and follicular dendritic cells (FDCs) in mice (59, 60, 63, 78), we investigated whether CR1/2 expression on hematopoietic derived B cells or non-hematopoietic derived FDCs is required for the development of an anti-KEL alloantibody response in the absence of $CD4^+$ T cells. To test this, recipients that were deficient in CR1/2 specifically on non-hematopoietic cells were generated by lethally irradiating and reconstituting CR1/2 KO recipients with B6 bone marrow that expresses the congenic marker CD45.1 to distinguish between recipient (CD45.2) and donor hematopoietic derived cells. As lethal irradiation depletes hematopoietic cells, transplantation of wild type B6 bone marrow into CR1/2 KO recipients permitted CR1/2 deficiency specifically on non-hematopoietic cells. Following reconstitution, recipients were depleted of $CD4^+$ T cells and subsequently transfused with KEL RBCs.

Despite the presence or absence of $CD4^+$ T cells, recipients specifically deficient in CR1/2 expression on non-hematopoietic cells generated a robust anti-KEL IgM response (Figure 6A). Likewise, similar to the ability of B6 recipients expressing CR1/2 on both hematopoietic and non-hematopoietic cells to generate an anti-KEL IgG response

in the absence of $CD4^+$ T cells (Figure 1), depletion of $CD4^+$ T cells in recipients specifically deficient in CR1/2 expression on non-hematopoietic cells failed to alter the anti-KEL IgG response following KEL RBC transfusion (Figure 6A). These results demonstrate that non-hematopoietic cell expression of CR1/2 is not required to induce a T cell independent antibody response to KEL. Rather, these findings suggest that hematopoietic cell expression of CR1/2 may be required to mount a $CD4^+$ T cell independent anti-KEL IgG response following exposure to KEL RBCs.

To test whether hematopoietic expression of CR1/2 is important for the generation of a T cell independent anti-KEL antibody response, B6 recipients were conversely transplanted with CR1/2 KO bone marrow. Transplantation of CR1/2 KO bone marrow into B6 recipients permitted the generation of hematopoietic cells specifically deficient in CR1/2. To determine CR1/2 KO bone marrow engraftment and complete loss of recipient hematopoietic cells, B6 recipients that express the congenic marker CD45.1 were utilized. Following engraftment, recipients were treated with PBS or depleted of $CD4^+$ T cells, and subsequently transfused with KEL RBCs. Depletion of $CD4^+$ T cells in recipients that lacked CR1/2 specifically on hematopoietic cells failed to generate a significant anti-KEL IgG response (Figure 6B). These results indicate that non-hematopoietic expression of CR1/2 alone does not induce a $CD4^+$ T cell independent antibody response to the KEL antigen on transfused KEL RBCs. Rather, these findings demonstrate that CR1/2 expression on hematopoietic cells is required for the ability of KEL RBCs to induce a $CD4^+$ T cell independent antibody response.

Discussion

In contrast to ABO(H) carbohydrate blood group antigens, the vast majority of clinically relevant RBC antigens are polymorphic proteins or glycoproteins capable of inducing both cellular and humoral adaptive immunity. Consistent with this, alloimmunization to some of these antigens has been shown to correlate with specific HLA allotypes (14, 15, 21), indicating a requirement for $CD4^+$ T cell help. Moreover, recent studies have demonstrated that RhD (15), Jk^a (96), and Kell-derived peptides (51) can be presented by distinct HLA variants to T lymphocytes (19, 51). Accordingly, we hypothesized that the blood group antigen KEL induces a $CD4^+$ T cell dependent antibody response. However, in contrast to previous notions and the model RBC antigen HOD, MHC-dependent and MHC-independent $CD4^+$ T cell help was found to be dispensable for the formulation of a functional KEL alloimmune response. Moreover, despite C3 fixation on transfused KEL RBCs, the generation of anti-KEL alloantibodies was also found to occur independent of C3 and CR1/2. However, unexpectedly, C3 and CR1/2 were found to regulate whether humoral immunity to KEL occurred independent of $CD4^+$ T cell help, with the absence of C3 or CR1/2 resulting in a $CD4^+$ T cell dependent alloantibody response to transfused KEL RBCs. These findings do not exclude the possibility that when C3 is present KEL can activate $CD4^+$ T cells and that these $CD4^+$ T cells can contribute to KEL alloimmunization. Rather, the current study demonstrates that the complement pathway may play a novel immunological role as a molecular switch that is capable of regulating whether humoral immunity to the KEL antigen occurs through a T cell independent or dependent pathway.

Classically, T cell independent antigens can be divided into T cell independent

type I or type II antigens. Mitogenic stimuli (i.e. LPS) that can elicit non-specific or polyclonal activation of B cells via pattern recognition receptor (i.e. toll like receptors) ligation are traditionally classified as T cell independent type I antigens (97). Though proficient at direct B cell activation, T cell independent type I antigens are inefficient inducers of isotype switching and affinity maturation, both characteristics conventionally associated with T cell dependent humoral immune responses. In contrast, T cell independent type II antigens are typically distinguished as carbohydrate antigens with highly organized, repetitive structures (i.e. bacterial capsular polysaccharides) that extensively cross-link B cell receptors, thereby directly delivering strong activation signals to B cells and rapid downstream induction of low affinity antibodies, predominately IgM (98). While exposure to T cell independent type II antigens can also result in production of IgG, the mechanism(s) by which class switching occurs in response to a T cell independent type II antigen remains less clear. Previous studies demonstrate that complement receptor ligation in conjunction with B cell receptor cross-linking and/or cytokines derived from bystander $CD4^+$ T cells can be important in antibody production following antigen exposure (56-58). However, in contrast to this classical paradigm wherein antigens are thought to intrinsically be T cell dependent or independent, the ability of KEL to induce a $CD4^+$ T cell dependent humoral response only in the absence of C3 or CR1/2 demonstrates that, in addition to biochemical features of a given immunogen, extrinsic immune factors like complement may actually possess the unique ability to regulate whether the same antigen induces an antibody response through a T cell independent or dependent pathway.

As complement receptor expression, and in particular CR1/2, is part of the

CD19/CD81 activation complex that potentiates B cell signaling after B cell receptor ligation (63, 73, 74, 76, 83, 99, 100), our findings corroborate earlier studies that demonstrate that regardless of whether an antigen biochemically fits into the current classifications of T cell dependent or T cell independent immunogens, the complement receptor is critical for optimal B cell receptor signaling. Moreover, these results demonstrate that the biochemical nature of an immunogen alone may not be sufficient to determine whether an antigen will induce a T cell independent or dependent antibody response. Though most antigens are certainly classified as T cell dependent or independent based on key biochemical properties, our current data indicate that the ability of early antibodies formed against a particular antigen to induce complement activation may specifically dictate whether the exact same antigen induces IgG antibody formation through a T cell independent or dependent process. These results therefore suggest a novel regulator that can govern the immune pathway through which an antibody response occurs. While the exact mechanism(s) by which C3 drives IgG antibody formation in the absence of $CD4^+$ T cell help is outside the scope of the current study, these findings demonstrate that complement may possess the ability to regulate the antibody response to antigens like KEL.

Although HLA restricted Kell peptides have been shown to activate $CD4^+$ T cells *in vitro* (51), and therefore suggest that $CD4^+$ T cell activation may occur in the human setting, given our present findings, it remains possible that these activated Kell reactive $CD4^+$ T cells may be regulated by the presence or absence of C3. While clinically anti-KEL IgG antibodies are not known to fix complement, as antibody formation is typically not monitored in real-time, whether IgM antibodies can fix complement during the

development of an anti-KEL antibody response remains unknown. Nevertheless, whether Kell$^+$ RBCs retain the capacity to induce a CD4$^+$ T cell independent antibody response in human subjects remains unknown; the presence of identifiable Kell reactive CD4$^+$ T cells does not necessarily mean these CD4$^+$ T cells are required to induce an alloantibody response to Kell. While several RBC alloantigens appear to be associated with particular HLA alleles (14-23), implicating a potential requirement for CD4$^+$ T cell help, alloimmunization to other RBC antigens fail to demonstrate a similar association with HLA type (101), suggesting that CD4$^+$ T cell independent antibody responses may also occur following exposure to other RBC alloantigens. The potential ability of a RBC alloantigen to induce antibodies through CD4$^+$ T cell independent but complement dependent pathway is important, as CD4$^+$ T cells have been postulated as a possible target in the prevention of RBC alloimmunization. However, whether these findings recapitulate clinical KEL alloimmunization or simply provide a model to study complement-dependent CD4$^+$ T cell independent mediated alloimmunization to other RBC alloantigens in general remains unknown. Regardless, this model system provides a unique opportunity to examine the key immune factors that may regulate alloantibody formation against clinically relevant RBC alloantigens. Furthermore, these findings may also suggest that therapies targeted toward CD4$^+$ T cells alone may not be sufficient to regulate other undesirable immune responses, as may occur in certain autoimmune diseases, as it is possible that complement activation in some patients with self-reactive antibodies may likewise promote the formation of autoantibodies through a CD4$^+$ T cell independent pathway, thereby bypassing regulatory networks that may normally rely on CD4$^+$ T cells (102-108).

While a variety of traditional model antigens, including hen egg lysozyme, ovalbumin, NP and keyhole limpet hemocyanin have been extensively studied to define the role of key immune populations in the development of a humoral immune response and immune function in general (60, 109-111), to our knowledge, much less is known about the potential role of common immune players, such as $CD4^+$ T cells and complement, in the formation of antibodies against RBC alloantigens like KEL. The results presented herein suggest that C3 may govern a novel immune response and in so doing dictate whether humoral immunity occurs through a $CD4^+$ T cell independent or dependent process. While characterization of how C3 may regulate the immune pathway through which an antibody response to KEL can occur is outside the scope of the current study in part due to a lack of commonly available immunological tools, such as BCR transgenics, TCR transgenics and tetramers typically used to study other model antigens, the current results provide important insight into a novel role of complement in the development of humoral immunity.

In summary, the main immunological constituents currently considered to be important in the induction of RBC alloimmunization are helper $CD4^+$ T cells and B cells. However, the results of the present study demonstrate that the innate immune factor complement may be an important immune factor that dictates whether the same antigen elicits a $CD4^+$ T independent or dependent antibody response. These results therefore suggest that different RBC alloantigens may possess the ability to induce alloantibody formation through distinct immune pathways. As these findings are in a murine model, testing the hypothesis in a human setting would be required before any clinical conclusions can be drawn. Nonetheless, the relevancy of the current findings to human

medicine is that they suggest an underappreciated immunological modulator of RBC alloimmunization.

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Figures and Legends:

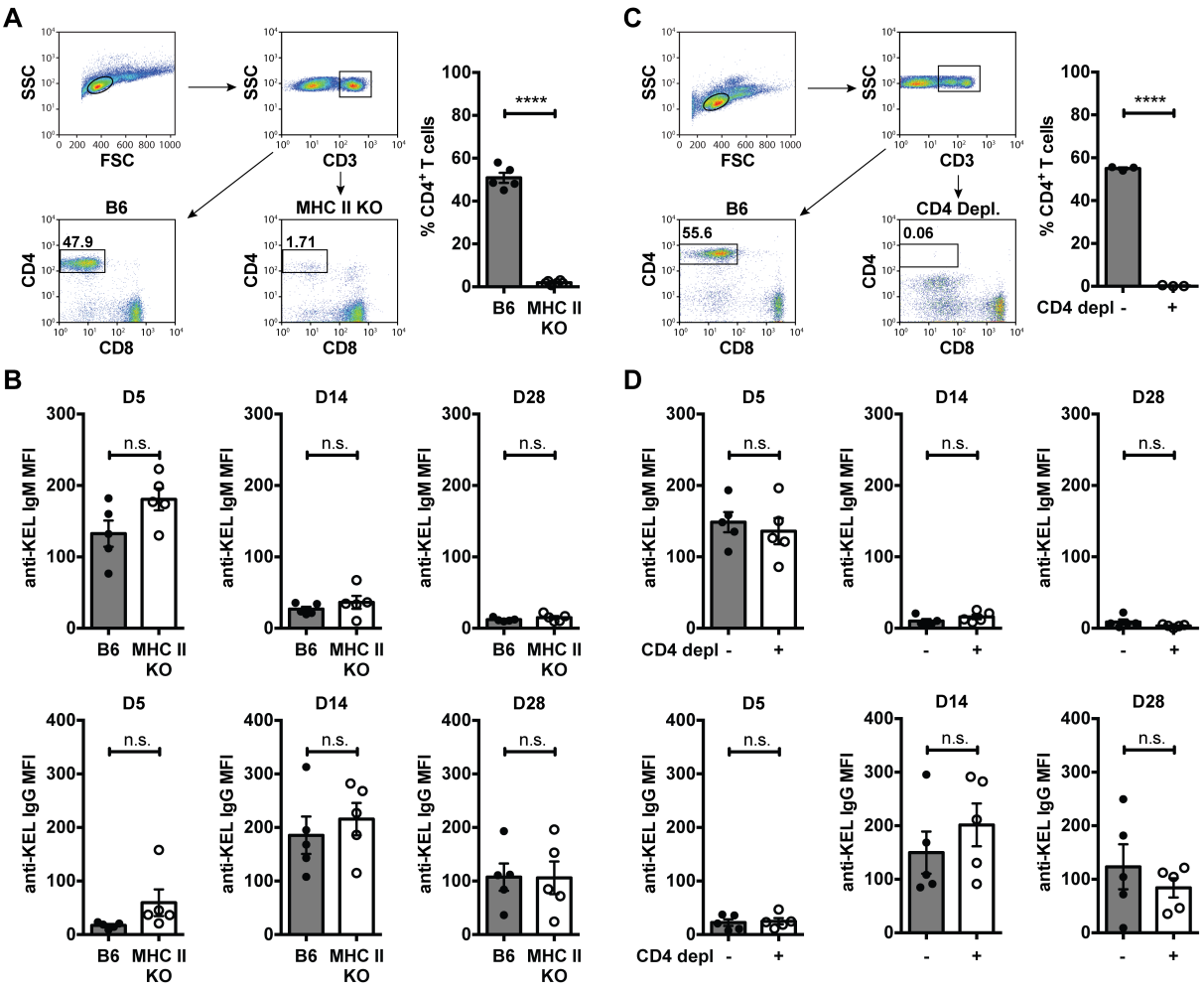


Figure 1. CD4$^+$ T cell help is not required to mediate KEL alloimmunization. (A) Representative flow plots with graphical illustration of percent splenic CD3$^+$ CD4$^+$ T cells in wild type B6 and CD4$^+$ T cell deficient MHC Class II KO (MHC II KO) recipients. **(B)** KEL specific alloantibodies in B6 and MHC Class II KO (MHC II KO) recipients transfused with KEL RBCs. **(C)** Representative gating strategy, as well as graphical demonstration of percent splenic CD3$^+$ CD4$^+$ T cells in KEL negative B6 recipients treated with PBS (-) or monoclonal anti-mouse CD4 depleting antibody (+). **(D)** Anti-KEL alloantibody formation in PBS (-) or monoclonal anti-mouse CD4 depleting antibody (+) treated B6 recipients transfused with KEL RBCs. Serum was

collected on days 5 (D5), 14 (D14) and 28 (D28) post transfusion in panels **(B)** and **(D)**, and serological analysis for anti-KEL IgM and IgG alloantibodies in panels **(B)** and **(D)** was examined by indirect immunofluorescence staining using KEL and B6 RBCs. The mean fluorescence intensity (MFI) in panels **(B)** and **(D)** were derived from normalizing the MFI of serum samples incubated with KEL RBCs to background control B6 RBCs. Error bars represent mean $\pm$ SEM. Statistics were generated using an unpaired student t-test. All panels show representative data from experiments reproduced at least 3 times, with at least 5 mice per group. ****, $p < 0.0001$ and n.s. indicates not statistically significant.

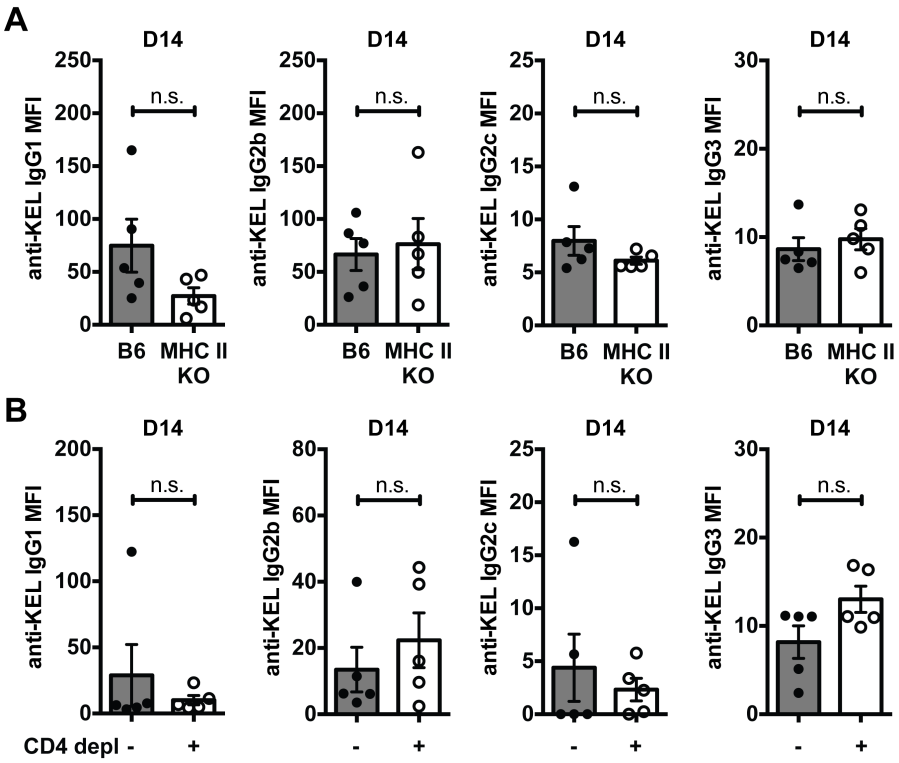


Figure 2. CD4$^+$ T cells fail to influence anti-KEL IgG subclass following KEL RBC transfusion. **(A)** Representative graphical presentation of anti-KEL IgG subtypes 14 days (D14) post transfusion of KEL RBCs into wild type B6 recipients or CD4$^+$ T cell deficient MHC Class II KO (MHC II KO) recipients transfused with KEL RBCs. **(B)** Anti-KEL IgG subtype analysis in the serum of wild type B6 recipients treated with PBS (-) or monoclonal anti-mouse CD4 depleting antibody (+). Serological examination for anti-KEL IgG subtypes (IgG1, IgG2b, IgG2c, and IgG3) in panels **(A)** and **(B)** was examined at 14 days (D14) post transfusion through indirect immunofluorescence staining using KEL and B6 RBCs. The MFI in both panels was computed by normalizing the MFI of samples incubated with KEL RBCs to background control B6 RBCs. Errors bars represent mean $\pm$ SEM. Statistics were generated using unpaired student t-test. All panels illustrate representative data from experiments reproduced 3

times, with each repeat comprising of at least 5 mice per group. n.s. indicates not statistically significant.

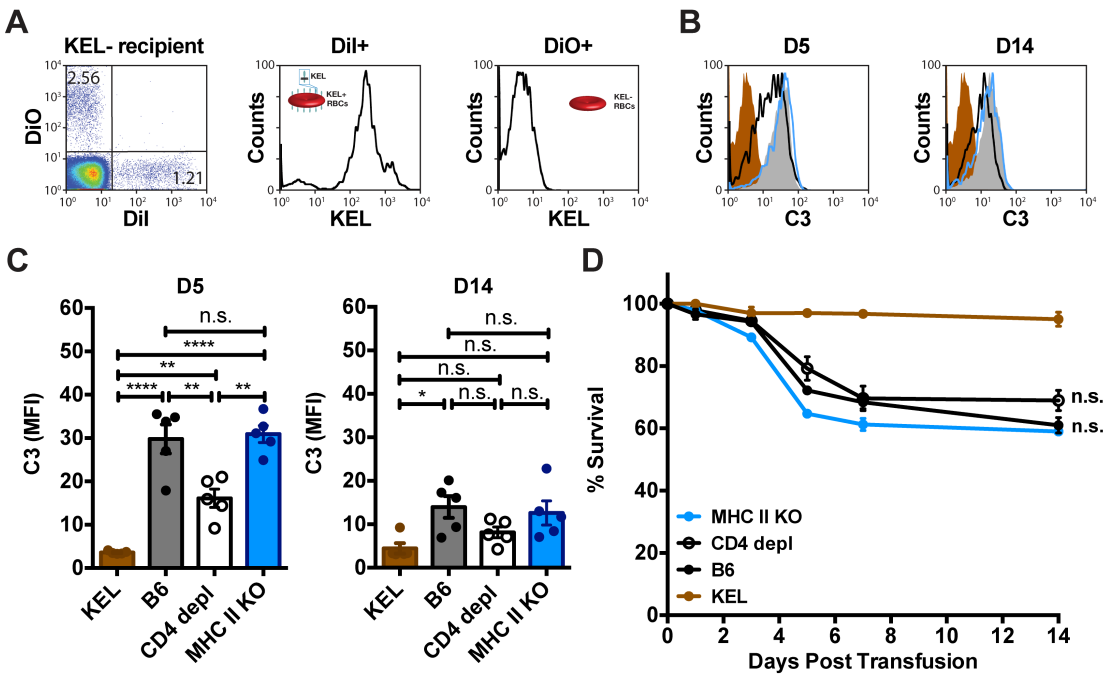


Figure 3. KEL specific alloantibodies generated in the absence of CD4$^+$ T cells induce C3 fixation and clearance of transfused KEL RBCs. KEL and CD4$^+$ T cell deficient MHC Class II KO (MHC II KO) recipients, as well as B6 recipients treated with PBS (B6) or monoclonal anti-mouse CD4 depleting antibody (CD4 depl.) were transfused with a 1:1 mixture of KEL and B6 RBCs labeled with DiI or DiO, respectively. **(A)** Flow cytometric gating strategy used to specifically examine transfused KEL-DiI RBCs. **(B)** Representative histograms of C3 fixation on KEL-DiI RBCs 5 (D5) and 14 (D14) days post transfusion. KEL positive recipients are depicted as a solid **brown** shade, PBS (B6) treated B6 recipients are illustrated as a **grey** shade, CD4$^+$ T cell depleted recipients are signified as a **black** line and CD4$^+$ T cell deficient MHC Class II KO recipients as a **blue** line. **(C)** Graphical demonstration of C3 fixation on KEL-DiI RBCs 5 (D5) and 14 (D14) days post transfusion. The MFI in panel **(C)** was calculated by normalizing the MFI of KEL-DiI RBCs stained for C3 to the background control KEL-DiI RBCs incubated with a streptavidin secondary only. C3 fixation on transfused

KEL-DiI RBCs is defined as a MFI above that of KEL-DiI RBCs from background control KEL recipients. **(D)** KEL RBC survival 3, 5, 7, and 14 days post transfusion. Survival of KEL RBCs was determined by comparing a ratio of KEL-DiI RBCs to B6-DiO RBCs. Errors bars represent mean $\pm$ SEM. Statistics were generated using One-way ANOVA with Tukey's post-test **(C)** or an unpaired student t-test **(D)**. All panels show representative data from experiments reproduced 3 times, with each repeat consisting of 5 mice per group. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$ and n.s. indicates not statistically significant.

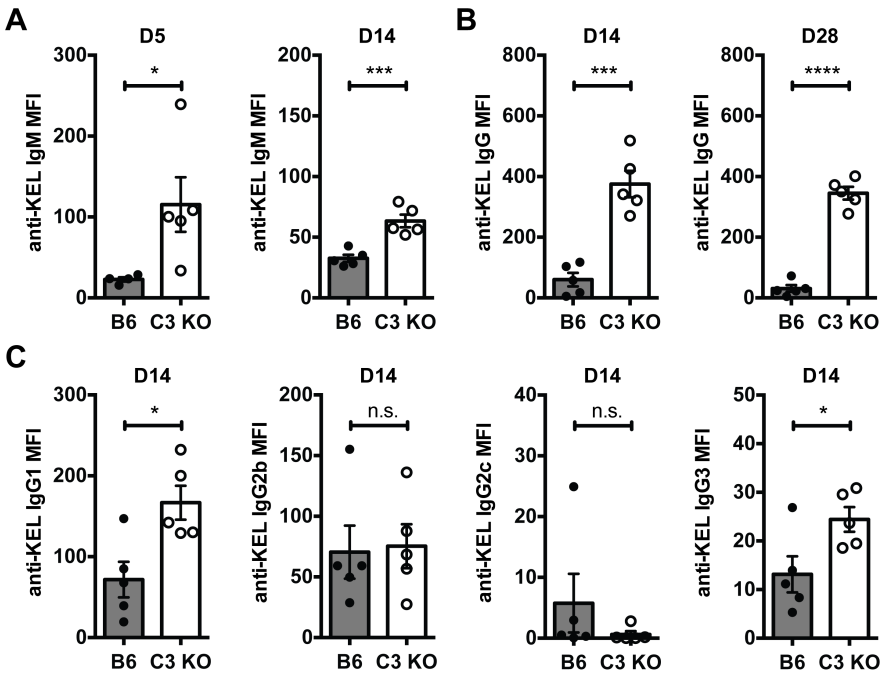


Figure 4. C3 KO recipients generate an enhanced anti-KEL antibody response following KEL RBC transfusion. Wild type B6 and C3 KO recipients were transfused with KEL RBCs. Serum was collected and evaluated for the development of **(A)** anti-KEL IgM on days 5 (D5) and 14 (D14) and **(B)** anti-KEL IgG on days 14 (D14) and 28 (D28) post-transfusion. **(C)** The development of IgG1, IgG2b, IgG2c and IgG3 antibodies was additionally assessed on 14 days (D14) post-transfusion. Errors bars represent mean $\pm$ SEM. Statistics were generated using an unpaired student t-test. All panels show representative data from experiments reproduced at least 3 times, with 5 mice per group. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$ and n.s. indicates not statistically significant.

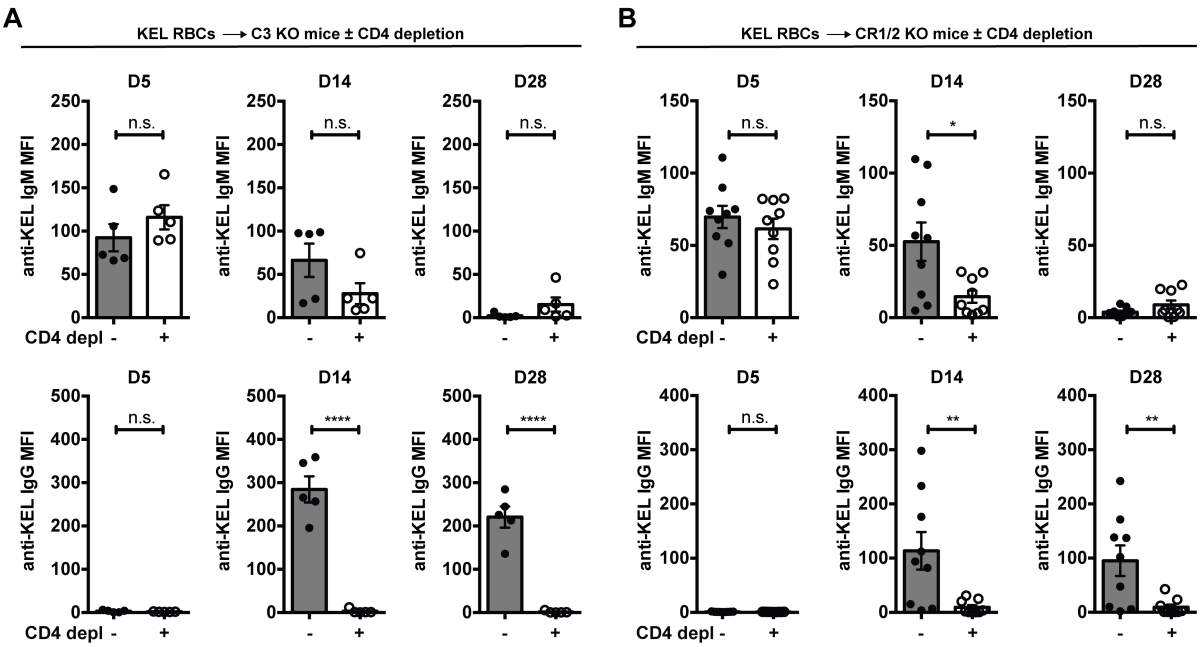


Figure 5. Alloantibody formation to KEL in the absence of C3 and CR1/2 is dependent on CD4$^+$ T cells. C3 KO **(A)** or CR1/2 KO **(B)** recipients were treated with PBS (-) or monoclonal anti-mouse CD4 depleting antibody (+) 4 and 2 days prior to transfusion of KEL RBCs. Serum was collected and evaluated for the development of anti-KEL IgM and IgG on days 5 (D5), 14 (D14) and 28 (D28) post-transfusion. Errors bars represent mean $\pm$ SEM. Statistics were generated using an unpaired student t-test. All panels show representative data from experiments reproduced at least 3 times, with 5 mice per group. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$ and n.s. indicates not statistically significant.

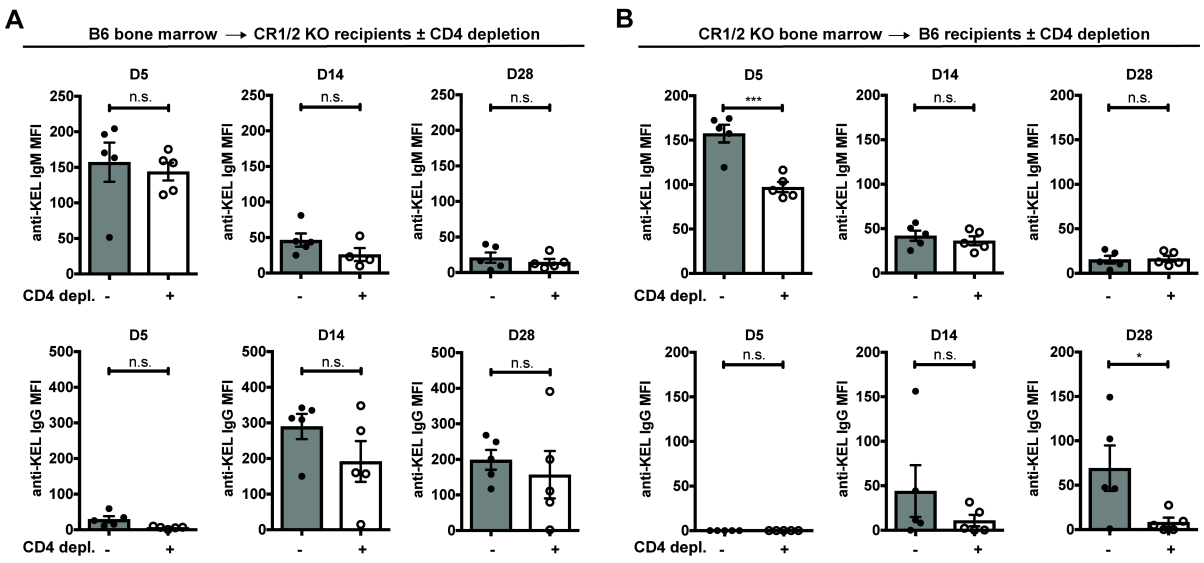
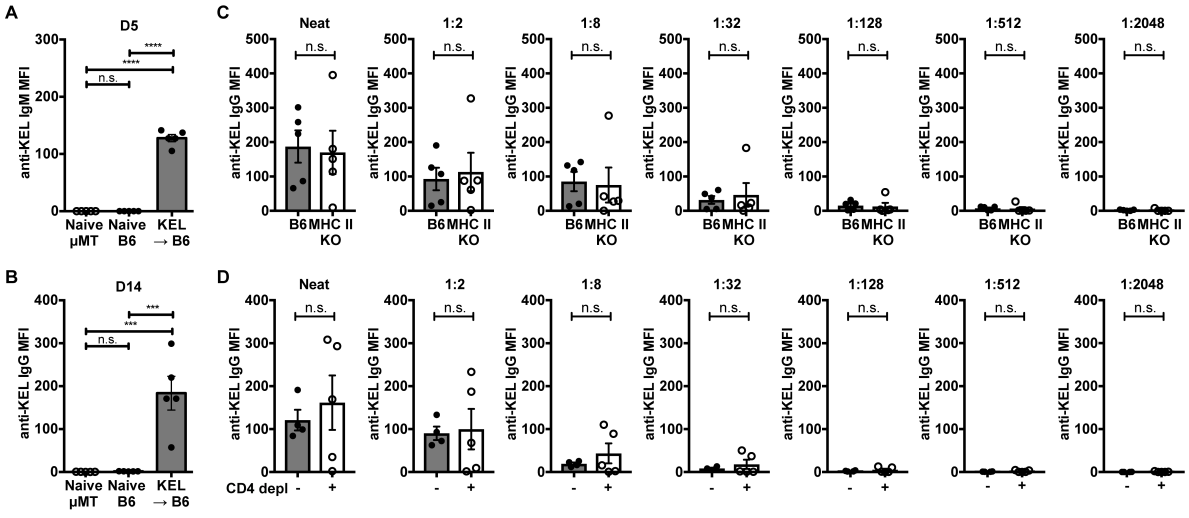
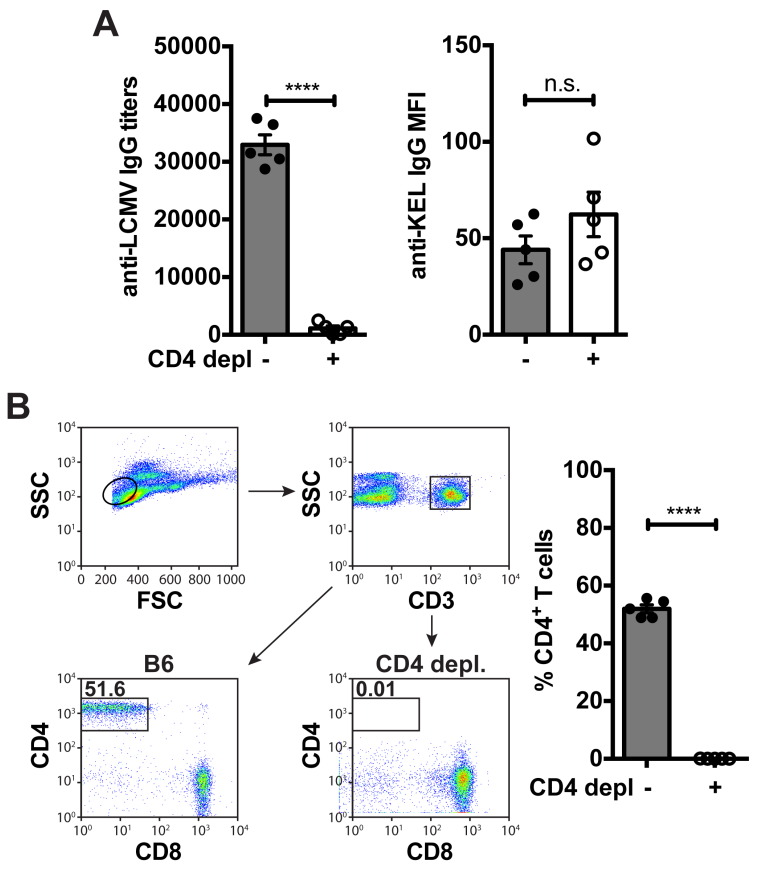


Figure 6. Hematopoietic expression of CR1/2 rescues development of anti-KEL IgG in the absence of CD4$^+$ T cells. **(A)** CD45.2$^+$ CR1/2 KO recipients were lethally irradiated and transplanted with bone marrow derived from wild type CD45.1$^+$ B6 mice. Following engraftment, recipients were treated with PBS (-) or a monoclonal anti-CD4 depleting antibody (+), and examined for anti-KEL IgM and IgG production days 5 (D5), 14 (D14) and 28 (D28) post-transfusion of KEL RBCs using by indirect immunofluorescence staining. **(B)** Wild type CD45.1$^+$ B6 recipients were lethally irradiated and transplanted with bone marrow from CD45.2$^+$ CR1/2 KO mice. Following engraftment, recipients were treated with PBS (-) or monoclonal anti-mouse CD4 depleting antibody (+) and subsequently transfused with KEL RBCs. Serum was collected on 5 (D5), 14 (D14) and 28 (D28) days post-transfusion, and tested for anti-KEL IgM and IgG using indirect immunofluorescence staining. Error bars represent mean $\pm$ SEM. Statistics were generated using an unpaired student t-test. All panels show representative data from experiments reproduced at least 2 times, with 5 mice per group. *, $p < 0.05$; ***, $p < 0.001$ and n.s. indicates not statistically significant.



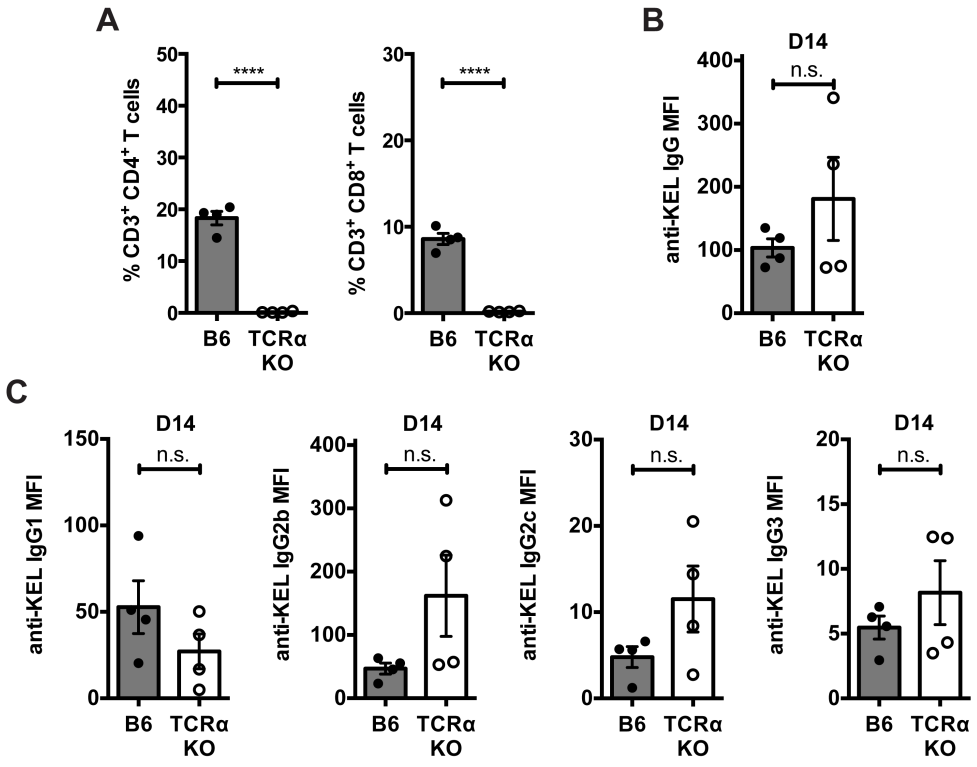
Supplementary Figure 1. Anti-KEL alloantibody class switching occurs independent of CD4$^+$ T cell help. **(A-B)** Graphical presentation of anti-KEL antibodies in the presence or absence of KEL RBCs into wild type B6 recipients or naïve recipients deficient in B cells (μMT). Serological examination for anti-KEL IgM **(A)** and IgG **(B)** was examined at 5 (D5) and 14 days (D14) post transfusion, respectively, through indirect immunofluorescence staining using KEL and B6 RBCs. **(C)** KEL specific IgG titration for serum isolated from B6 and MHC Class II KO (MHC II KO) recipients transfused with KEL RBCs. **(D)** Titration of anti-KEL IgG formation in PBS (-) or monoclonal anti-mouse CD4 depleting antibody (+) treated B6 recipients transfused with KEL RBCs. Serum was collected on day 14 post transfusion in panels **(C)** and **(D)**, and serological analysis for anti-KEL IgG alloantibodies in panels **(C)** and **(D)** was examined by indirect immunofluorescence staining using KEL and B6 RBCs. The MFI in all panels was computed by normalizing the MFI of samples incubated with KEL RBCs to background control B6 RBCs. Errors bars represent mean $\pm$ SEM. Statistics were

generated using one-way ANOVA with a post Tukey's test in panels **(A-B)** and an unpaired student t-test in panels **(C-D)**. ***, $p < 0.001$; ****, $p < 0.0001$ and n.s. indicates not statistically significant.

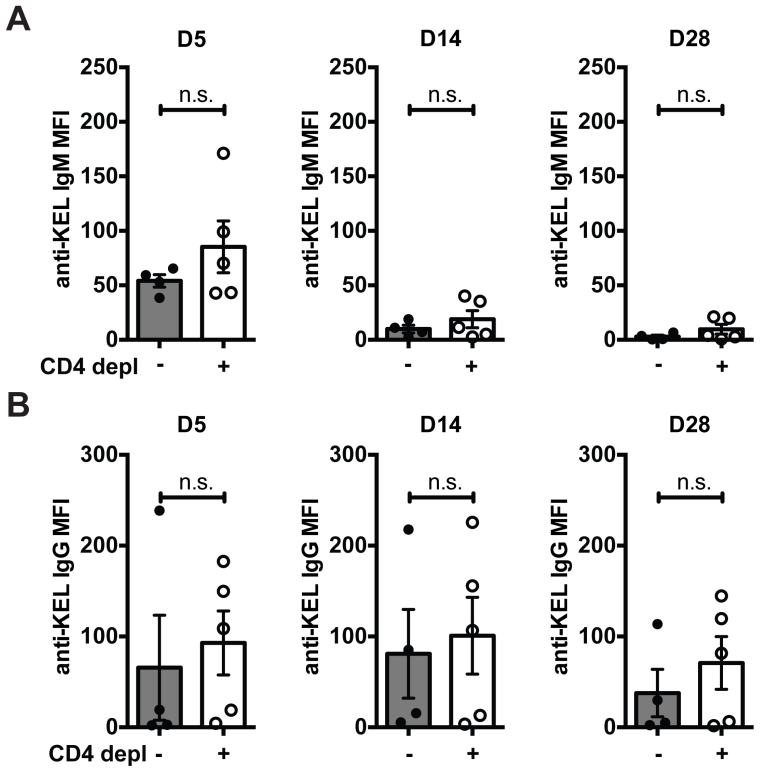


Supplementary Figure 2. Treatment with anti-CD4 depleting antibody prevents anti-LCMV antibody formation. **(A)** B6 recipients were treated with PBS (-) or monoclonal anti-mouse CD4 depleting antibody (+) 4 and 2 days prior to LCMV (clone: 13) infection or transfusion of KEL RBCs. Serum was collected 15 days post infection or transfusion. ELISA was used to examine sera for anti-LCMV IgG, and indirect immunofluorescence staining using KEL and B6 RBCs was performed to test for anti-KEL IgG antibodies. **(B)** Representative flow plots with graphical illustration of percent peripheral blood $CD3^+$ $CD4^+$ T cells in KEL negative B6 recipients treated with PBS (-) or monoclonal anti-mouse CD4 depleting antibody (+). Error bars represent mean $\pm$ SEM. Statistics were generated using an unpaired students t-test. All panels show

representative data from experiments reproduced at least 3 times, with 5 mice per group. ****, $p < 0.0001$ and n.s. indicates not statistically significant.

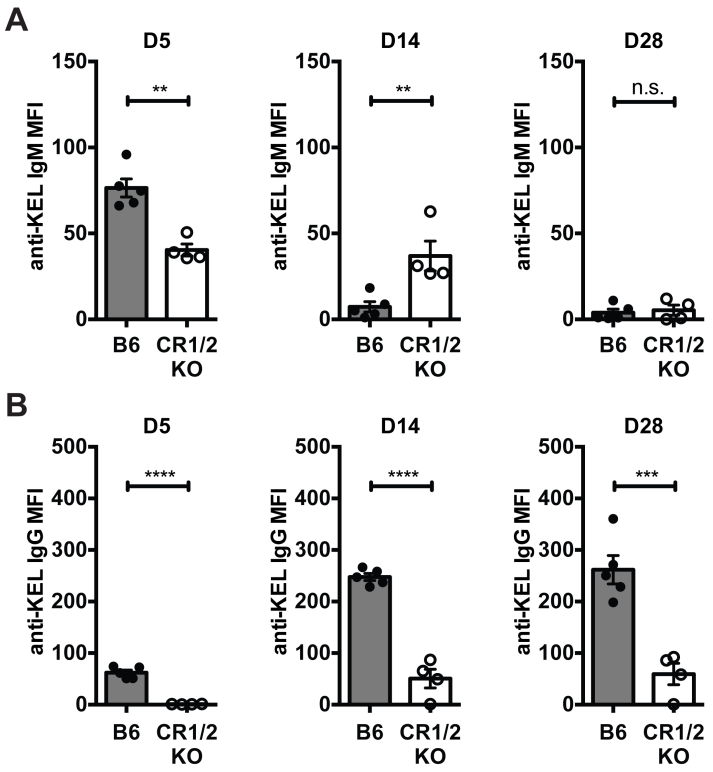


Supplementary Figure 3. TCRα KO mice generate an anti-KEL alloantibody response following KEL RBC transfusion. (A) Percent $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ T cells in the peripheral blood of TCRα KO recipients. **(B)** Serological analysis of anti-KEL IgG (day 14 = D14) in KEL negative B6 recipients and TCRα KO recipients transfused with KEL RBCs. **(C)** Anti-KEL IgG subtype (IgG1, IgG2b, IgG2c, and IgG3) analysis in the serum of wild type B6 and $CD4^+$ T cell deficient TCRα KO recipients 14 days (D14) post transfusion of KEL RBCs. The MFI in both panels was computed by normalizing the MFI of samples incubated with KEL RBCs to background control B6 RBCs. Error bars represent mean $\pm$ SEM. Statistics were generated using a student's t-test. All panels show data from experiments reproduced at least 3 times, with 3-4 mice per group. ****, $p < 0.0001$ and n.s. indicates not statistically significant.



Supplementary Figure 4. Recipients deficient in C5 generate an anti-KEL antibody response independent of CD4$^+$ T cells. C5 KO recipients were administered PBS (-) or a monoclonal anti-mouse CD4 depleting antibody (+) and subsequently transfused with KEL RBCs. Ant-KEL IgM **(A)** and IgG **(B)** was examined on 5 (D5), 14 (D14) and 28 (D28) days post transfusion through indirect immunofluorescence staining using KEL and B6 RBCs. The MFI in both panels was computed by normalizing the MFI of samples incubated with KEL RBCs to background control B6 RBCs. Errors bars represent mean $\pm$ SEM. Statistics were generated using unpaired student t-test. All

panels illustrate representative data from experiments reproduced 2 times, with each repeat comprising of at least 5 mice per group. n.s. indicates not statistically significant.



Supplementary Figure 5. Alloantibody response to KEL is decreased in the absence of CR1/2. B6 and CR1/2 KO recipients were transfused with KEL RBCs. Ant-KEL IgM **(A)** and IgG **(B)** was examined on 5 (D5), 14 (D14) and 28 (D28) days post transfusion through indirect immunofluorescence staining using KEL and B6 RBCs. Normalizing the MFI of samples incubated with KEL RBCs to background control B6 RBCs generated the MFI in both panels. Errors bars represent mean $\pm$ SEM. Statistics were generated using unpaired student t-test. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$ and n.s. indicates not statistically significant.

Chapter 6
Discussion

Although red blood cell (RBC) transfusions are a common life-saving intervention in the setting of blood loss and hemoglobinopathies, they are not without risk. RBC alloantibodies occur when antigenic differences exist between RBC donor and recipient. Indeed, even a single alloantigenic difference may lead to the development of RBC alloantibodies against a particular RBC alloantigen. Although antigen matching protocols, which include genotyping and serological testing, can minimize the risk of alloantibody formation, in emergency settings, antigen-mismatched transfusions may be unavoidable. Although transfusion of antigen-positive blood into an antigen-negative individual can be avoided, in some cases, there is no treatment to prevent the development of anti-RBC alloantibodies. While some patients are repeatedly exposed to RBC alloantigens through chronic transfusions and factors, such as recipient inflammatory state (1), have been associated with responder status, given that other patients do not respond in the same setting, the immunologic mechanism(s) governing why some patients develop alloantibodies while others do not is not well understood.

As a result, in this dissertation, we sought to determine the potential mechanisms governing RBC alloimmunization in an attempt to potentially mitigate the development of these alloantibodies. In particular, we demonstrated how antibody binding to RBCs induces changes in levels of surface RBC antigen, termed antigen modulation, which may affect the development of these alloantibodies. Alloantibodies against minor RBC antigens and further examination of antigen modulation as a mechanism in the development of these alloantibodies are important for several reasons:

(i) RBC alloantibodies developed to minor antigens during a mother's first pregnancy can cross the placenta in subsequent pregnancies causing hemolytic disease of the fetus and newborn (discussed in Chapters 2-3).

(ii) Presence of alloantibodies against RBC alloantigens can make it difficult to find compatible blood for future transfusions. This is especially important in the context of sickle cell anemia where chronic or life-long transfusion support is the one of the only viable therapies in these patients. However, not all transfused individuals respond to donor RBC antigens. Unfortunately, it is not well understood why some patients become alloimmunized while others do not (discussed in Chapter 4).

(iii) Examining the development of these RBC alloantibodies can provide fundamental insight into the regulation of the adaptive immune system (discussed in Chapter 5).

The studies presented in this dissertation provide further insight into the development of RBC alloantibodies, for which no prophylactic therapy currently exists to actively prevent the development of RBC alloantibodies against RBC antigens other than RhD. Additionally, the data presented here show that these model RBC antigens can be a potential tool for not only examining the development of alloantibodies against RBC antigens, but also to better understand the interaction between innate and adaptive immunity.

Antibody-mediated immunosuppression

Through the studies presented in this dissertation (Chapter 2), we demonstrated that monoclonal antibodies could induce antibody-mediated immunosuppression (AMIS) via antibody-induced antigenic changes. Furthermore, the ability of monoclonal antibodies to induce antigen changes occurred independent of activating Fcγ receptors. Additionally, AMIS to RBC alloantigens is antigen-specific, as alloantibodies to one antigen only caused AMIS and antigen modulation to that specific antigen (Chapter 3). These results indicate that antigen modulation may be a surrogate for identifying potential antibodies that may induce AMIS. However, several factors limit the implications of our findings as a potential screening method. First, we only studied one RBC antigen type with monoclonal antibodies that induce antigen modulation and AMIS. We cannot rule out the possibility that monoclonal antibodies against a particular RBC antigen may induce AMIS without antigen modulation or, alternatively, may induce antigen modulation without AMIS. It will be important to determine whether AMIS and antigen modulation are inextricably linked for other RBC antigens as well.

Our results suggest a mechanism of action initiated by the Fab portion of the antibody, rather than the Fc portion, given that AMIS and antigen modulation occurred independent of activating Fcγ receptors in the HOD RBC antigen model. In contrast to previous studies indicating that antigen modulation, to other RBC antigens, is at least partially dependent on activating Fc receptors (2-5), our findings suggest that that the mechanism of AMIS and antigen modulation may differ by the particular antigen. Regardless, previous studies have also suggested that different IgG subclasses, as well as different properties of the Fc portion of the antibody, may impact the ability of antibodies to induce AMIS. For example, Rh immune globulin (RhIg), polyclonal antibody from

pooled serum, is currently the only prophylaxis capable of actively preventing the development of anti-RhD alloantibodies. Furthermore, although RhIg is derived from pooled serum from either women alloimmunized against RhD through transfusion and pregnancy or men who have been purposely alloimmunized against RhD, purposeful alloimmunization is not a safe or ethical practice for development of RhIg-like prophylaxis against other RBC antigens. Therefore, to find an alternative to pooled serum from RhD alloimmunized donors, several studies have examined properties of monoclonal anti-RhD alloantibodies in their ability to induce AMIS. Although anti-RhD IgG1 initiated phagocytosis more than anti-RhD IgG3, anti-RhD IgG3 induced lysis of RBCs more readily than anti-RhD IgG1 (6, 7), suggesting that even antibodies with the same specificity but different subclasses could cause different immunological outcomes.

Similar to RhIg, infusion of intravenous immune globulin (IVIG), which consists of IgG from thousands of healthy donors per batch, can dampen the immune response. IVIG has been used as a treatment for a variety of autoimmune conditions, such as Kawasaki disease, Guillain-Barre and immune thrombocytopenic purpura (ITP) (8). Despite its effectiveness, little is known regarding the precise molecular mechanisms governing its ability to induce AMIS. However, investigation into IVIG and the qualities of IgG within IVIG that allow it to be effective in suppressing the immune response in autoimmunity have identified several properties that warrant investigation in RBC alloimmunization as well. For example, treatment of IVIG with an enzyme that removes sialic acid from IgG revealed the importance of sialylation in mediating the anti-inflammatory effects of IVIG in a murine model of inflammatory arthritis (9). Likewise, enhancement of sialylation increased the ability of IVIG to mediate anti-inflammatory

activity (10). As the glycosylation patterns of IgG can impact the affinity for Fc receptors, with sialylated and fucosylated Fc regions having reduced affinity for Type I Fc receptors (11, 12), which include FcγRs I-IV, these studies suggest that monoclonal antibodies against RBC antigens that have more sialylation may have an increased ability to induce AMIS potentially in an activating Type I Fc-independent manner. We may therefore be able to manipulate the glycosylation patterns of antibodies to identify potential antibodies capable of inducing AMIS.

In addition to the sialylation of the Fc portion of potential AMIS-inducing antibodies, previous studies have also examined how the glycosylation patterns of AMIS-inducing antibodies potentially impact the immune cell types and receptors engaged, which may therefore indicate how AMIS-inducing antibodies function in different settings. In studies examining the anti-inflammatory properties of IVIG, mice deficient in inhibitory FcγRIIB developed ITP after administration of a monoclonal anti-platelet antibody even in the presence of IVIG. In contrast, in wild-type mice, IVIG was protective against the development of ITP (13), suggesting a role for FcγRIIB in mediating the anti-inflammatory effect of IVIG. In a subsequent study of IVIG in a model of murine inflammatory arthritis, mice deficient in SIGN-R1, a Type II Fc receptor and C-type lectin receptor on macrophages, were unresponsive to IVIG administration (14, 15). However, blocking antibodies specific to other receptors on macrophages did not interfere with the ability of IVIG to induce immunosuppression (14, 16-18). Furthermore, as adoptive transfer of splenocytes from mice treated with IVIG into naïve mice protected against IVIG only when the recipients expressed FcγRIIB, Anthony *et al.* proposed a model in which IVIG binding to SIGN-R1 on macrophages caused an anti-

inflammatory phenotype that led to upregulation of FcγRIIB (Figure 1). These results suggest that SIGN-R1 may be a potential target receptor on macrophages to examine for antibody binding against RBC antigens to better understand why some antibodies induce AMIS while others do not. However, as mice deficient in FcγRIIB have been previously shown to undergo AMIS following exposure to RBCs expressing hen egg lysozyme (HEL), ovalbumin, and human Duffy (HOD RBCs) in the presence of polyclonal anti-HEL antibody (19), it is unclear what role FcγRIIB may play in the development of AMIS and how this may affect the dependence of AMIS to RBC antigens on SIGN-R1 expression.

Despite other potential mechanisms, such as antibody glycosylation and immune receptor engagement that may equally impact AMIS, our studies demonstrated that antigen modulation may also be one potential mechanism governing the ability of antibodies to induce AMIS. Furthermore, the findings presented in this dissertation demonstrate that antigen modulation and AMIS are antigen-specific, as exposure to an antigen-specific antibody only induces antigen modulation and AMIS to that RBC antigen when more than one antigen is expressed on the RBC surface (Chapter 3). However, the precise molecular mechanisms governing antigen modulation and AMIS remain unclear, as activating Fcγ receptors were dispensable for antigen modulation and AMIS to occur when RBCs only expressed the HOD antigen, but have been shown to be important in antigen modulation and AMIS to other model RBC antigens (2, 4, 5). Regardless of whether one or more RBC antigens were expressed on RBCs, antigen appeared to be completely removed from the RBC surface, as shown through western blot analysis (Chapters 2-3). Additionally, our results demonstrated that each RBC

antigen retains its individual properties even when expressed on the same RBC, as antibodies developed following transfusion of RBCs that express another model antigen, KEL, induce complement fixation on KEL RBCs, but antibodies developed following transfusion of HOD RBCs do not induce robust complement fixation on HOD RBCs. Because one molecular pathway important in antigen modulation involves complement fixation on the KEL RBC surface (3) and antibodies directed toward the HOD antigen do not robustly fix complement post-transfusion on HOD nor HOD x KEL RBCs, it is difficult to assess the role of complement in antigen modulation and AMIS following exposure to HOD RBCs. Therefore, studies utilizing another model RBC antigen, KEL, in which antibodies developed following transfusion of KEL RBCs induce complement fixation post-transfusion, allowed for further examination of the role of C3 in RBC alloimmunization.

Complement fixation and RBC alloantibody development

Several mechanisms have been suggested to explain why some patients develop alloantibodies against RBC antigens. One mechanism proposed is inflammation at the time of transfusion (1, 20, 21), which can prime the immune system for later development of RBC alloantibodies. Additionally, inflammation can also break tolerance to RBC antigens that previously did not elicit an alloantibody response, suggesting that inflammation can both be an initiator and driver of alloantibody development (22). In addition to inflammation, another potential driver for the development of RBC alloantibodies that may synergistically interact with inflammation to drive RBC alloantibody development, could be complement fixation. Although RBCs have been

shown to fix complement during hemolytic transfusion reactions (23-25), and complement has been shown to be important in clearance and antigen modulation of KEL RBCs following passive administration of anti-KEL polyclonal antibody (3), studies in this dissertation elucidated the role of complement in the development of *de novo* RBC alloantibodies. Previous studies challenging mice with *Streptococcus pneumoniae* (26-28), herpes virus (29) and bacteriophage (30) demonstrated that complement component 3 (C3) is required for a robust humoral immune response. Opsonization of antigen by complement is important for phagocytosis by macrophages and dendritic cells via complement receptor 3 and 4 expression, leading to presentation via MHC class II to $CD4^+$ T cells (31-33), as well as antigen loading on follicular dendritic cells (FDCs) by complement receptors 1 and 2 (CR1/2) (34). Recycling of antigen throughout the germinal center has been shown to be important for the repetitive stimulation of B cells through B cell receptors (BCRs) and development of high affinity antibody through affinity maturation via somatic hypermutation in the germinal center (35). Despite the described role of C3 in FDC antigen acquisition, through the interaction of C3-opsonized antigen with CR1/2 receptors on FDCs, our results show that mice deficient in C3 (C3 KO mice) actually develop increased anti-KEL IgM following KEL RBC exposure. These mice are capable of class switching and form increased IgG following exposure to KEL RBCs. We also found that one potential mechanism for this increased antibody response was due to increased antigen post-transfusion compared to B6 mice, suggesting increased antigen availability to the BCR (Chapter 4). Given that a certain threshold of antigen is necessary to stimulate B cells to develop into antibody-secreting cells, these results indicate that increased antigen availability from the absence of complement-

mediated antigen modulation may therefore contribute to B cell activation through reaching the threshold of antigen necessary for B cell activation to occur.

As antigen density and organization are important to the development of a robust antibody response (36-40), these results suggest that the absence of C3 may lead to organization the membrane in such a way that antigen density is maximized, potentially allowing increased activation of the BCR. This may potentially occur through C3's effect on the RBC antigen itself but also through its impact on membrane fluidity, given that C3 fixation has been previously shown to increase the rigidity of the RBC membrane following fixation (41, 42). Consequently, the absence of C3 fixation may result in increased membrane fluidity that increases the ability of antigen to cluster, thereby increasing antigen density to reach the threshold of B cell activation. Furthermore, our results illustrate that complement contributes to antigen removal in B6 mice, as shown when RBC membranes were isolated and probed for KEL expression by western blot analysis. These western blot results show that complement alters antigen availability by selective removal of the target antigen without altering the overall membrane integrity, given that there were no changes in Ter119, another RBC antigen, and furthermore, no detectable changes in RBC size or shape by flow cytometry (Figure 2).

That B cells may become activated even in the absence of complement deposition is further illustrated by examining the role of complement receptors in the development of anti-KEL alloantibodies. Classically, CR1/2 are selectively expressed on FDCs and B cells in mice, involved in the development of germinal center reactions and are a member of the B cell activation complex. In addition to its importance in germinal center formation, CD21 (CR1) is a member of the B cell activation complex, along with CD19

and CD81 (37, 39, 43, 44). Antigen recognition by the BCR can induce receptor clustering and association of CD81 with CD21 and CD19 (45). BCR activation and clustering results in a downstream signaling cascade, resulting in phosphorylation of transcription factors involved in NF-κB signaling and cell proliferation (46-48).

Classically, BCR recognition of antigen with co-receptor clustering has been shown to occur with C3-opsonized antigen, resulting in concurrent binding of antigen to the BCR and C3 to CR1. However, our results show that C3 does not seem necessary for an antibody response to the KEL antigen to occur, given that C3 KO mice exhibit increased anti-KEL IgM and IgG (Chapter 4). In contrast, CR1/2 does seem to be important in anti-KEL antibody development, as mice deficient in CR1/2 expression (CR1/2 KO) exhibit decreased anti-KEL IgG development (Chapter 5). Bone marrow chimera experiments further revealed that B cell expression of CR1/2 is important for the development of robust anti-KEL IgG, whereas CR1/2 on FDCs is not necessary for development of anti-KEL IgG (Chapter 5). Taken together with our previous data that C3 is able to modulate the KEL antigen on the RBC surface (Chapter 4), our findings suggest that in the absence of C3, no direct binding between C3 and CR1/2 occurs. However, given the increased antibody response observed in C3 KO mice, we interpret our findings to suggest that B cell signaling with clustering of co-receptors, including, but not limited to, CR1/2, may occur when sufficient KEL antigen on the RBC surface is able to allow for BCR clustering. Conversely, in the absence of CR1/2, CR1/2 KO mice had decreased development of an anti-KEL alloantibody response, indicating that C3 modulates the KEL antigen levels even in the absence of CR1/2, thereby leading to the decreased KEL alloantibody response observed in CR1/2 KO mice. However, in the presence of C3, anti-

KEL alloantibodies, which develop following transfusion of KEL RBCs, can fix C3, leading to not only antigen modulation, but also interaction with CR1/2 and development of a T-independent alloantibody response. Taken together, these results suggest that while CR1/2 is necessary for a robust anti-KEL IgG response, C3, which typically binds to murine CR1/2 on FDCs and B cells, is not. Together, these results suggest a model of B cell activation during the development of anti-KEL antibody response in which KEL either interacts with the BCR directly in the absence of C3 binding to CR1/2, which may be sufficient to induce non-complement-mediated clustering of the BCR and co-receptors to activate B cells through non-complement-mediated intracellular activation of the CD21 signaling cascade.

To better understand how C3KO mice are able to develop an enhanced humoral antibody response to the KEL RBC antigen but not to other antigens, it will be important to determine the nature of this B cell activation, such as whether there is a difference in proliferation or activation marker expression between C3 KO and B6 mice in response to exposure to KEL RBCs through utilizing *in vitro* culture and mitogenic stimulation. Together with ELISPOTs of cells harvested from the spleen and bone marrow to determine the quantity of antibody-secreting cells, the B cell activation results will reveal whether the enhanced antibody response observed for C3 KO mice results in increased number of antibody-secreting cells or rather increased B cell activation and antibody production from a particular subset of B cells. In addition to ELISPOTs, development of a B cell transgenic against KEL will allow for more precise examination of the activation state of antigen-specific B cells. As previous studies have demonstrated that C3-deficient mice are unable to form robust germinal centers (26, 30-32, 49-54), immunofluorescence

of splenic follicles will indicate whether normal germinal center formation occurs compared to wild-type mice and whether previous results utilizing C3-deficient mice revealed an antigen-specific defect in germinal center formation rather than a general defect.

Negative regulatory role of complement and implications for vaccination

Our results that complement can negatively regulate the antibody response to an antigen by affecting the level of target antigen on the RBC surface may have significant implications for not only understanding the development of RBC alloantibodies in transfusion medicine, but also in developing vaccines to antigens. Part of vaccinology has focused on increasing the immunogenicity of a vaccine so that the components of the vaccine resembles the pathogen enough to stimulate an antibody response but not resembling the pathogen to such an extent that negative side effects occur. As such, recombinant proteins, virus-like particles and virosomes that incorporate virus proteins into a liposome are several methods that were developed to utilize the properties of antigen presentation to enhance immunogenicity of the vaccine (55, 56). Some current models of vaccine development assume that antibody and complement may facilitate antigen presentation, leading to the development of an immune response against an antigen. For example, while some studies have sought to decrease complement activation to avoid drug delivery clearance (57), other studies found that complement activation on Pluronic-coated surfaces results in enhanced immunogenicity to ovalbumin following administration of ovalbumin conjugated to nanoparticles (58), presumably by complement deposition leading to enhanced phagocytosis and antigen presentation.

However, our data suggests that in cases of certain types of antigen, complement fixation actually leads to decreased antibody responses through modulation of the surface antigen, which in the setting of vaccination, could account for the poor immunogenicity of some vaccines. From our results, it is possible that upon immunization, complement binding to a particular vaccine delivery method could actually lead to a reduced antibody response against an antigen. Therefore, based on our data, it may be necessary to individually assess whether complement negatively regulates the antibody response to an antigen. Furthermore, it is possible that a particular vaccination delivery method (i.e. liposome) facilitates complement fixation that may interfere with the development of an antibody response and, therefore, an alternative delivery method should be considered.

In our model of RBC alloimmunization, the KEL-positive donor and KEL-negative recipients only differ in the expression of KEL on an otherwise syngeneic RBC. In contrast, in the delivery of viral or bacterial particles during vaccination, there are many inherent differences between pathogen and host. Furthermore, delivery of viral or bacterial particles containing pathogen-associated molecular patterns (PAMPs) that engage pattern recognition receptors, may further contribute to activation of the complement cascade in its classically-described role as an adjuvant in the development of an immune response. Therefore, it is equally possible that our findings regarding the negative regulatory role of complement are unique due to the way in which RBC antigens are presented on an otherwise syngeneic RBC surface, in contrast to pathogens or vaccines that may contain multiple foreign components to readily engage the innate immune system. Furthermore, RBCs circulate in the blood and traffic to the marginal zone within the spleen (59, 60), as opposed to the ability of some vaccines to deliver

antigen for presentation to dendritic cells or B cells in the subcapsular sinus of the lymph nodes as well (58, 61, 62). Consequently, it is possible that the differential localization of antigen following immunization, along with activation of the immune system by PAMPs, may lead to more opportunities for antigen presentation that are less impacted by the negative regulation of complement observed in our studies with RBCs.

Complement as a switch governing T cell independent antibody responses

We observed a T-independent antibody response to KEL as supported by $CD4^+$ T cell depletion studies in B6 mice, as well as mice deficient in $CD4^+$ T cells, such as MHC class II KO and TCRα KO mice. However, we also observed that C3 KO mice mounted an unexpected increase in anti-KEL IgM and IgG, with C3 KO mice exhibiting increased IgG1 compared to B6 mice (Chapter 5). Since $CD4^+$ T cell production of IL-4 is important for IgG1 class switching by murine B cells (63-65), we next investigated whether complement may potentially drive the T-independent antibody response to KEL observed in B6 mice. Utilizing a combination of $CD4^+$ T cell depletion and bone marrow chimeras, these studies demonstrated that C3 KO and B6 mice exhibit a different requirement for $CD4^+$ T cell help (Chapter 5). More specifically, while B6 mice are able to develop anti-KEL IgG in the absence of $CD4^+$ T cells, C3 KO mice are completely reliant on $CD4^+$ T cells to develop anti-KEL IgG. These results suggest that C3 fixation on the KEL RBC surface confers T-independence. Given that previous studies have shown that C3 fixation can be involved in both T-independent and T-dependent antibody responses (26, 30, 32, 49-54, 66, 67), these results instead demonstrate that C3 may be a requirement for T-cell independence to a RBC antigen.

These results show that an innate immune factor possesses the ability to regulate adaptive immunity to a given antigen. Although it has been long-appreciated that complement can interface with the development of an adaptive immune response, such as in germinal center formation or loading of antigen on FDCs (68-71), taken together, this data shows that C3 fixation can actually not only determine whether an antibody response to an antigen will require T cell help, but also act as a novel toggle between T-independent and -dependent antibody responses. As both classically-defined T-independent or T-dependent antigens have been shown to fix complement, we do not interpret these results to suggest that complement fixation alone is predictive of T cell independence versus dependence. We only present this data as perhaps one explanation for the reliance on $CD4^+$ T cells observed for RBC antigens. In particular, despite the fact that KEL is a glycoprotein and is biochemically defined as a T-dependent antigen based on previous studies identifying the $CD4^+$ T cell epitopes for KEL (72), when KEL fixes complement, it actually elicits T-independent antibody responses. In contrast, HOD does not induce the same degree of complement fixation compared to KEL and is T-dependent, further illustrating that complement fixation below a necessary threshold on the RBC surface may lead to a reliance on $CD4^+$ T cell help for the development of RBC alloantibodies.

Given the differences in T cell requirement for the development of anti-KEL antibody responses in B6 and C3 KO mice, it will be interesting to determine whether complement fixation results in differential activation, proliferation or cytokine profiles associated with exposure to KEL RBCs. In a murine model of pulmonary allergy using infection with *Aspergillus fumigatu*s, absence of C3 resulted in suppression of Th2

effector functions with a reduction in eosinophils and IL-4 producing $CD4^+$ T cells (73). These results suggest that complement is important in the development of $CD4^+$ T cell Th2 activation. In contrast, in other studies, C5 incubation with dendritic cells, followed by co-culture with CD4 T cells resulted in Th1 cytokine production (74, 75), suggesting that complement may actually be important in the development of a Th1 $CD4^+$ T cell response. Together with our data demonstrating that C3's interaction with CR1/2 is important for a T-independent antibody response, it is evident that complement may play multiple roles in regulating the $CD4^+$ T cell response to antigenic challenge.

Depending on the antigen and the ability of antibody engagement of an antigen to induce complement fixation, our results and other studies indicate that the role of complement in the immune response may vary. The route of antigenic challenge is one potential reason for these different outcomes to complement activation. Intranasal infection of *Aspergillus fumigatu*s resulted in Th2 skewing (73), whereas intraperitoneal introduction of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) resulted in Th1 responses (76). Furthermore, intravenous administration of KEL RBCs resulted in a T-independent response. While these results may suggest that the route of administration may impact the relationship between complement and $CD4^+$ T cells, because there are other examples in which intranasal administration of bacteria results in skewing toward a Th1 response (76), the relationship between route of administration and T cell activation may be more complex than currently appreciated.

Although our data suggest that complement's interaction with complement receptors may regulate whether the antibody response to a RBC antigen is T-independent or -dependent, there are most likely other immune cell populations that are involved in

the development of RBC alloantibody responses. Because we have previously demonstrated in other studies that marginal zone B cells are important in the development of anti-KEL antibody responses, as transfusion of KEL RBCs into marginal zone B cell-deficient mice or mice depleted of marginal zone B cells results in a decreased anti-KEL alloantibody response, potential receptors that may play a role in the development of RBC alloantibody responses may include those that bind IgM, consisting of FcμR, a B cell-specific IgM receptor, along with Fcα/μ receptor (Fcα/μR), which binds IgA and IgM on macrophages, FDCs and both follicular and marginal zone B cells (77-80), collectively referred to as FCMRs. Previous studies have suggested that mice deficient in Fcα/μR have enhanced germinal center responses and development of IgG3 antibodies after immunization with a T-independent antigen, suggesting a negative regulatory role for Fcα/μR (81). In examining the humoral response to T-dependent antigens, mice deficient in an IgM-specific Fc receptor expressed only on B cells (FcμR) have also been shown to have decreased antibody development and germinal center formation after immunization with nitrophenyl-coupled chicken γ-globulin (NP-CGG) (82, 83), whereas in another study, FcμR-deficient mice had enhanced plasma cell formation to immunization with pneumococcus or NP conjugated to keyhole limpet hemocyanin (83, 84), suggesting that the negative regulatory role of FcμR to T-dependent antigens may potentially depend on the type of antigen and even the antigen dose, given that it has been shown that a higher dose of NP-CGG may overcome the antibody production deficit observed in FcμR-deficient mice (82).

However, previous studies have also observed that antigen retention to a T-independent antigen was complement-dependent even in Fcα/μR-deficient mice (81),

indicating that IgM binding to FCMRs and involvement in complement activation may work synergistically. Given our results showing that complement is a negative-regulator in the development of anti-KEL alloantibody responses and that complement deposition on the RBC surface correlates with deposition of IgM on the RBC surface (Chapter 4), it is possible that IgM binding to the RBC surface post-transfusion not only results in complement-binding that facilitates antigen removal, but may also engage FCMRs on B cells or FDCs, leading to the dampened immune response observed. However, in the absence of complement and the absence of complement-mediated antigen modulation, FCMRs may be able to cross-link the BCR, leading to enhanced B cell survival and thus further contributing to the enhanced antibody response in C3 KO mice observed. Further studies in Fcα/μR or Fcμ-deficient mice following exposure to KEL RBCs will better elucidate how these receptors may contribute mechanistically to not only antigen-modulation, but also anti-KEL alloantibody development.

Given that complement binding regulates whether the antibody response to KEL RBCs is T-independent or -dependent, we interpret these findings to suggest that complement binding may alter the immune pathway involved in the development of anti-RBC alloantibodies. Previous studies have indicated that resting T cells contain intracellular C3, C3aR and cathepsin L (CTSL) storages. CTSL cleaves C3 intracellularly at a slow rate to maintain T cell survival and mTOR activity (85, 86), suggesting a potential interplay between complement activation and mTOR activation, as well as T cell metabolism and homeostasis. Furthermore, although studies have described the interaction between complement receptors and lymphocyte activation in human T cells due to the restricted expression of CD46, with no known homologue in mice (85-93), it is

less clear how complement receptors and the mTOR pathway may interact with other cell types and in mice to regulate lymphocyte homeostasis. However, mTOR has been shown to be important in regulation of B cell homeostasis as well, as mice hypomorphic for mTOR or hypomorphic specifically in B cells have decreased ability to form germinal centers, develop high affinity antibodies or perform somatic hypermutation and class switch recombination (94). This notion of B cell regulation by mTOR is further supported by the fact that Blys, which is a survival factor for B cells has been found to promote B cell survival and function through 2 independent pathways that can compensate for each other, one of which is the Akt/mTOR pathway (95). mTOR has also been shown to be important in B cell interaction with T cells, as co-culture of T regulatory helper cells, which express FoxP3 along with T follicular helper (Tfh) cells markers such as CXCR5, with Tfh and B cells leads to a decrease in GLUT1 expression and IgG1 production. However, it is unclear from this data whether Tfh cells regulate B cells through the mTOR pathway specifically (96). Taken together, these previous findings suggest that mTOR is not only important in the regulation of T cell homeostasis due to complement activation, but mTOR may also play more of a pleiotropic role in B cell activation as well. Together with our findings showing that complement activation engages a T-independent pathway in the development of anti-KEL antibodies, perhaps C3 binds to complement receptors to activate mTOR in B cells to promote the development of anti-KEL antibodies. In the absence of complement, one possibility is that KEL is able to directly engage B cell receptors, leading to clustering of B cell receptors and complement receptors, thereby leading to the activation of mTOR and production of anti-KEL antibodies. In the absence of direct engagement of complement receptors through C3,

there may be an alternate pathway by which C3 KO mice are able to mediate an mTOR-independent but T-dependent development of anti-KEL antibodies. Further studies utilizing both mTOR depletion through rapamycin and $CD4^+$ T cell depletion in the presence or absence of complement will better determine how mTOR may be involved in the development of anti-KEL antibodies in the presence or absence of $CD4^+$ T cells, as well as complement.

In addition to the mTOR pathway, future studies of how complement may engage a different immune pathway independent of $CD4^+$ T cells in response to anti-KEL RBC exposure may also focus on the role of interferon (IFN) signaling, given more recent studies exploring the role of type I IFN signaling in the development of RBC alloantibodies. Type I IFNs have been shown to have both positive and negative regulatory roles in B cell development and activation, given that Type I IFNs have been shown to inhibit IL-7 induced growth and survival of B cell precursors *in vitro* and $CD19^+$ pro B cells *in vivo* (97). However, Type I IFNs have been shown to induce a state of partial B cell activation and increased resistance to Fas-mediated apoptosis in an *in vitro* model (98). In contrast, previous studies examining the role of Type I IFNs in relation to RBC alloimmunization specifically have shown that signaling through Type I IFNs is required for inflammation-induced anti-KEL alloimmunization (99) and that IFNAR1 expression on hematopoietic cells and B cells specifically are important in the development of anti-KEL alloantibodies (100). Given that aberrant IFN signaling has been reported in patients with autoimmune diseases (101-104) and there are elevated alloimmunization rates in patients with autoimmune diseases (1, 105, 106), it is likely that IFN signaling may play a complex and intimate role in the development of RBC

alloantibodies both in a healthy patient and potentially in the context of autoimmunity as well. However, it is currently unclear how complement deposition on the RBC surface may engage the Type I IFN pathway in a CD4-T cell independent manner to contribute to the development of RBC alloantibodies. Given that there has been reported intersection of the complement and Type I IFN pathway, interferon regulatory factor (IRF) signaling leading to Type I IFN production and mTOR activation, it is possible that these pathways together all contribute to driving the development of RBC alloantibodies in the absence of T cell help (107-109) (Figure 4). How these signaling pathways contribute to RBC alloantibody development, synergistically or individually, will require selective depletion studies in which the mTOR pathway or Type I IFN signaling are examined individually, before determining whether they can potentially act together to drive RBC alloantibody development, or even compensate for each other when either is absent.

General Conclusions

Transfusion of RBCs offers a life-saving intervention to not only treat patients during acute blood loss during trauma, but also serves as a therapy for patients suffering from hemaglobinopathies. Exposure to allogeneic RBCs, either through chronic RBC transfusions or pregnancy can lead to the development of RBC alloantibodies. These RBC alloantibodies can cause several complications, such as creating difficulty in finding compatible RBC units for future transfusions (1, 110, 111), or the development of hemolytic disease of the fetus in newborn (112, 113).

Despite the ability to prevent the development of RhD alloantibodies through administration of RhIg, there are still multiple unanswered questions regarding how RhIg

and potential monoclonal alternatives can suppress the development of *de novo* RBC alloantibodies. This dissertation describes the immunologic consequences of antibody binding to antigen on the RBC surface, resulting in antigen modulation. The findings presented in this dissertation suggest that antigen modulation is involved in not only AMIS, but also in the development of RBC alloantibodies. More specifically, antibodies to RBC antigens can potentially prevent the development of an alloantibody response against the target RBC antigen through rapidly inducing antigen modulation on the RBC surface in mice following exposure to allogeneic RBCs in an antigen-specific manner (Chapters 2-3). Furthermore, antigen modulation also plays a critical role in the development of the antibody response following exposure to RBCs, as complement was found to induce antigen modulation on the RBC surface, leading to a decreased humoral response in mice exposed to allogeneic RBCs. Absence of C3 led to a decrease in the rate of antigen modulation and an increased antibody response (Chapter 4) as a potential result of increased antigen exposure to BCRs.

Complement not only negatively regulated the antibody response following KEL RBC exposure by modulating the available antigen load, but also determined whether the antibody response to KEL was dependent on $CD4^+$ T cells thereby regulating the pathway through which the immune response occurs. The presence of C3 led to a T-independent alloantibody response, whereas the absence of C3 led to a T-dependent alloantibody response (Chapter 5). Taken together, the data in this dissertation support a model in which complement can not only surprisingly regulate the antibody response to an antigen, but also regulate the involvement of T cell help.

Although the current findings regarding antigen modulation and complement provide new insight regarding the development of alloantibody responses against RBC antigens, they do not address how this model would include antibodies developed against RBC antigens, or other antigens, that do not induce complement fixation nor antigen modulation post-transfusion. Furthermore, as the RBC alloantigen models in this thesis consist of either one or two antigenic differences on an otherwise syngeneic RBC, it is unclear how these results will translate to other antigen models and RBC transfusions in which many antigenic differences exist, as occurs clinically. Regardless, given the complexity of patient management in those with hemoglobinopathies and the lack of pharmacologic intervention, it is imperative to continue to try to understand the development of RBC alloantibodies in order to potentially prevent both their development and negative consequences once they have developed.

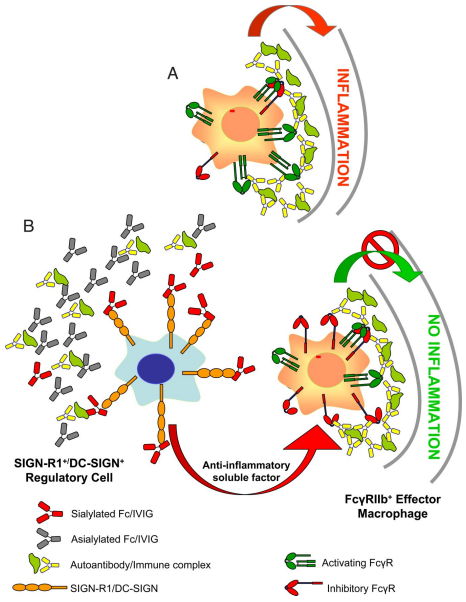


Figure 1: Potential mechanism of action of inhibitory antibodies in IVIG proposed by Anthony *et al.*, in which sialylated IgG in IVIG binds to SIGN-R1 on macrophages, leading to upregulation of FcγRIIB. SIGN-R1 may be a potential receptor to examine in AMIS against RBC antigens as well.

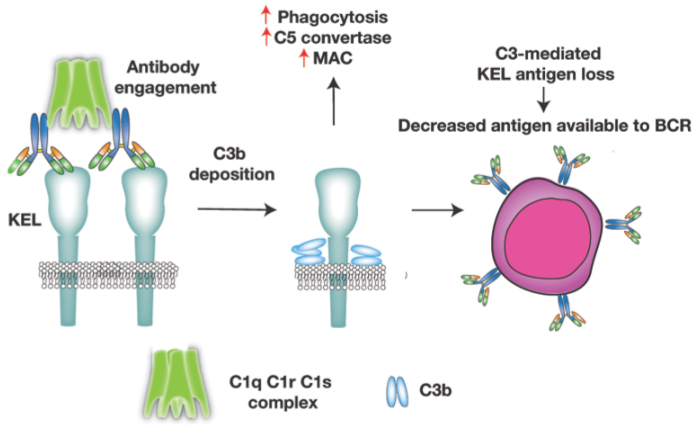


Figure 2: Proposed model of how antibody deposition and complement fixation on the RBC surface post-transfusion may affect antigen availability to the BCR.

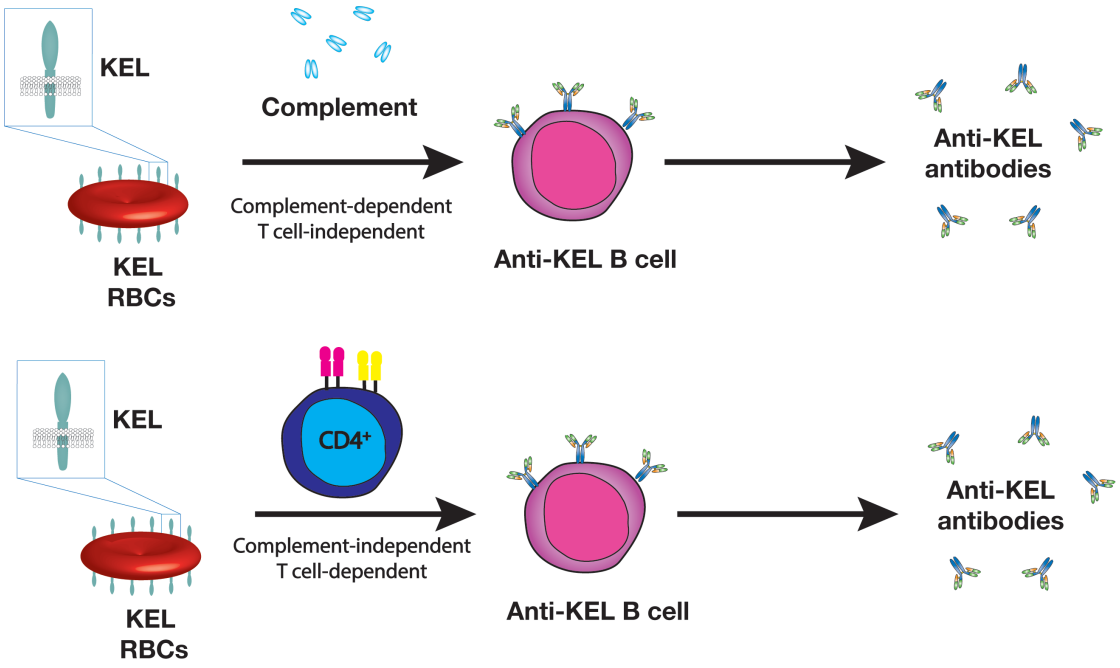


Figure 3: Proposed model of alloimmunization to the KEL antigen: Wild-type mice are able to develop anti-KEL IgG in the absence of $CD4^+$ T cells through complement fixation on the KEL antigen, which binds to CR1/2 on the B cells. However, in the absence of complement or complement receptors, C3 KO and CR1/2 KO mice are dependent on $CD4^+$ T cells to develop anti-KEL IgG.

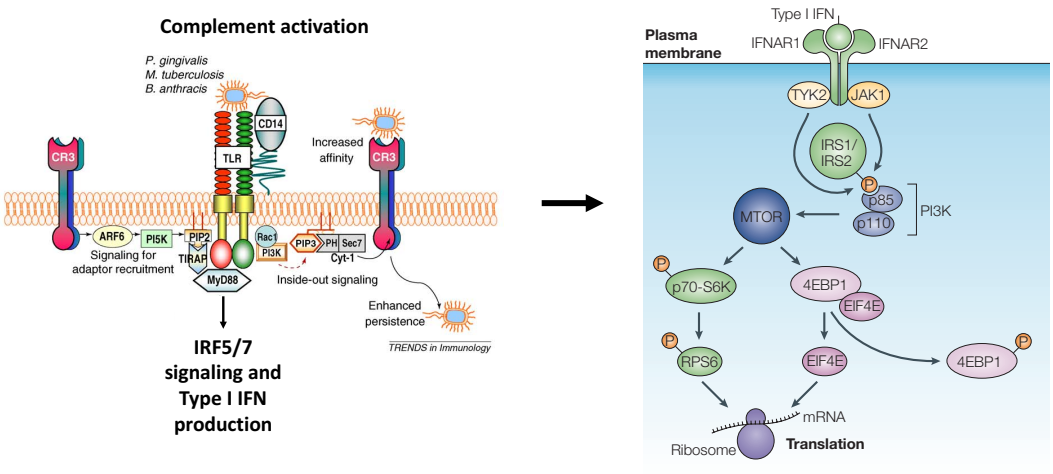


Figure 4: Potential convergence of various immune pathways in RBC alloimmunization. The complement pathway may contribute to IRF5 and IRF7 activation, leading to Type I IFN production through the MyD88 pathway. This Type I IFN may then be able to bind to IFNAR1 and 2, leading to mTOR activation downstream, contributing to antibody development in B cells.

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