

Complement serves as a switch between CD4⁺ T cell-independent and -dependent RBC antibody responses

Amanda Mener,¹ Seema R. Patel,¹ Connie M. Arthur,¹ Satheesh Chonat,² Andreas Wieland,³ Manjula Santhanakrishnan,⁴ Jingchun Liu,⁴ Cheryl L. Maier,¹ Ryan P. Jajosky,¹ Kathryn Girard-Pierce,¹ Ashley Bennett,¹ Patricia E. Zerra,¹ Nicole H. Smith,¹ Jeanne E. Hendrickson,⁴ and Sean R. Stowell¹

¹Center for Transfusion Medicine and Cellular Therapies, Department of Laboratory Medicine and Pathology, ²Aflac Cancer and Blood Disorders Center, Department of Pediatrics, and ³Department of Microbiology & Immunology, Emory University School of Medicine, Atlanta, Georgia, USA. ⁴Yale School of Medicine, Department of Laboratory Medicine, New Haven, Connecticut, USA.

RBC alloimmunization represents a significant immunological challenge for patients requiring lifelong 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 formation of alloantibodies to KEL, one of the leading causes of hemolytic transfusion reactions. Unexpectedly, our findings demonstrated 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 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. Repeated 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, 10). 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 (11). Unfortunately, no therapeutic modality currently exists that actively prevents humoral immunization to a RBC alloantigen (2, 11). 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 (e.g., Kell, Kidd, Duffy) are proteins or glycoproteins that are thought to lack fundamental biochemical properties of T cell-independent antigens (12). Rather, these non-carbohydrate blood group antigens have been uniformly thought to induce antibody responses through

Authorship note: AM and SRP contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

License: Copyright 2018, American Society for Clinical Investigation.

Submitted: April 13, 2018

Accepted: October 10, 2018

Published: November 15, 2018

Reference information:

JCI Insight. 2018;3(22):e121631.

<https://doi.org/10.1172/jci.insight.121631>

insight.121631.

CD4⁺ T cell help. Consistent with this, certain HLA class II alleles correlate with the risk of developing alloantibodies against some RBC antigens (13–22). This has led to CD4⁺ T cells becoming the primary focus of possible strategies designed to inhibit RBC alloimmunization in chronically transfused individuals (23–26). 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 (23). 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 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, 27–29). 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, 30–33). 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 (34–37), 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.

Results

KEL alloantibody formation occurs independent of CD4⁺ T cell help. To formally test the hypothesis that cognate T-B cell interactions are required for alloimmunization to KEL on transfused RBCs, WT C57BL/6 (B6) and MHC class II-KO (MHC II KO) recipients that were genetically deleted of MHC class II molecules and consequently deficient in CD4⁺ T cells (Figure 1, A and B) were transfused with a volume-adjusted equivalent of 1 human unit of KEL RBCs (7, 31–33, 38, 39). 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 and neat (undiluted) serum, a previously established method used clinically and experimentally to evaluate anti-RBC antibody formation (23, 31–33).

Surprisingly, transfusion of KEL RBCs into MHC II KO recipients generated an anti-KEL IgM and IgG response that was not statistically different from the antibody response observed in KEL RBC-transfused B6 WT recipients (Figure 1C). While indirect immunofluorescence staining represents the gold standard for examining antibody production in the field of transfusion medicine, 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 WT B6 recipients transfused with KEL RBCs (Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.121631DS1>). However, CD4⁺ T cell deletion is not complete in MHC II KO recipients (Figure 1B) (40). Thus, to additionally examine the role of CD4⁺ T cell help in KEL alloimmunization, B6 recipients negative for KEL were administered PBS or a CD4-depleting antibody (clone: GK1.5) 2 times, a day apart (Figure 1D). 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 1E).

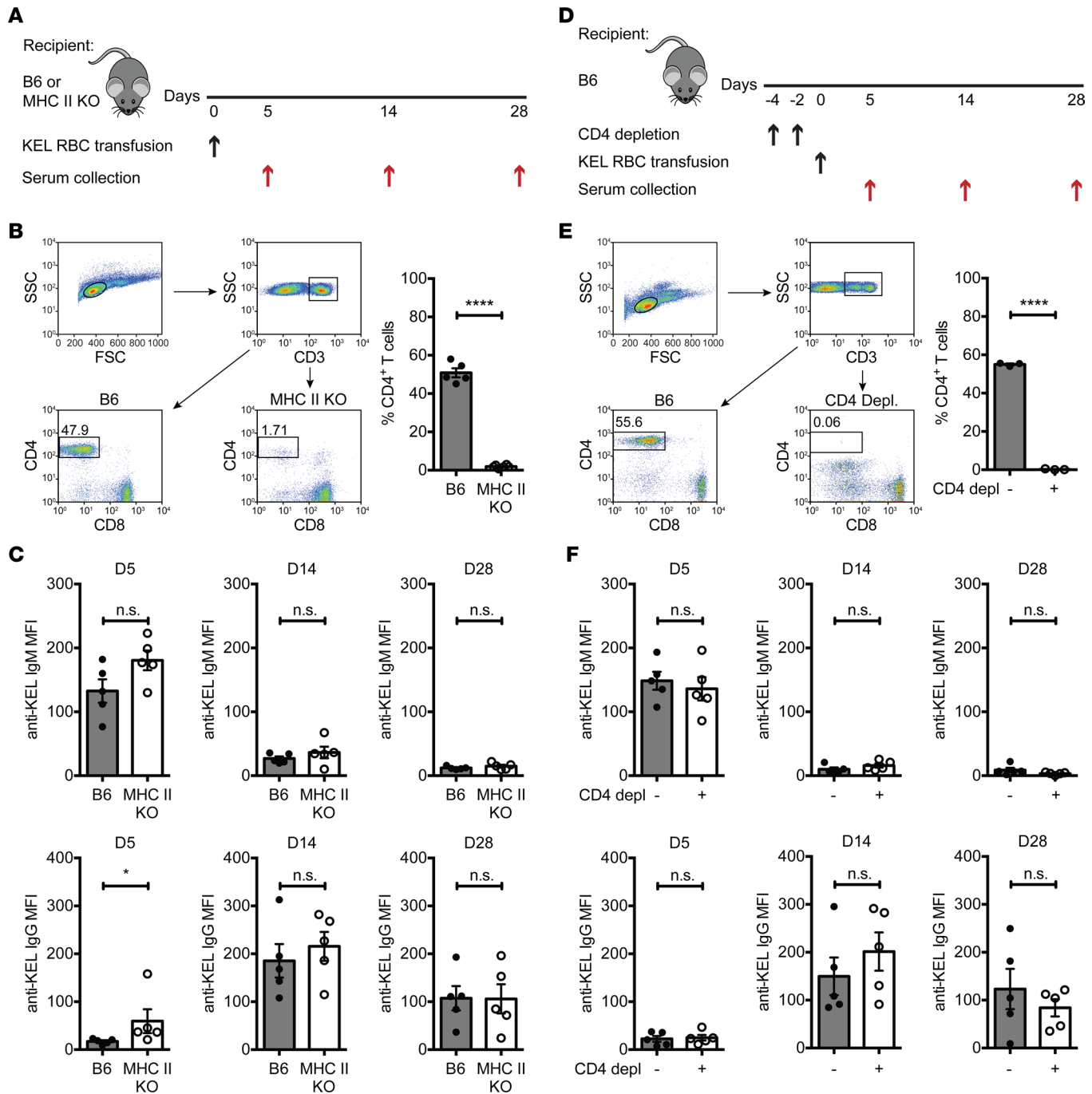


Figure 1. CD4⁺ T cell help is not required to mediate KEL alloimmunization. (A) Experimental schematic for **B** and **C**. (B) Representative flow plots with graphical illustration of percent splenic CD3⁺ CD4⁺ T cells in WT B6 and MHC class II KO (MHC II KO) recipients. (C) KEL-specific alloantibodies in B6 and MHC II KO recipients transfused with KEL RBCs. (D) Schematic of experimental outline for **E** and **F**. (E) Representative gating strategy, as well as graphical demonstration of percent splenic CD3⁺ CD4⁺ T cells in B6 recipients treated with PBS (-) or monoclonal anti-mouse CD4 depleting antibody (CD4 depl; +). (F) Anti-KEL alloantibody formation in PBS-treated (-) or monoclonal anti-mouse CD4 depleting antibody-treated (+) B6 recipients transfused with KEL RBCs. Serum was collected on days 5, 14, and 28 (D5, D14, and D28) after transfusion in **C** and **F**. Error bars represent mean \pm SEM. Statistics were generated using an unpaired Student's *t* test in **B** and **E**, and an unpaired Mann-Whitney *U* test in **C** and **F**. There were either 5 (**B**, **C**, and **F**) or 3 (**E**) mice per group. All panels show representative data from experiments reproduced 3 times. *****P* < 0.0001; n.s., not statistically significant.

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-treated (B6) recipients (Figure 1F). Similarly, serum serial titration demonstrated no statistically significant difference in anti-KEL IgG formation in CD4⁺ T cell-depleted or

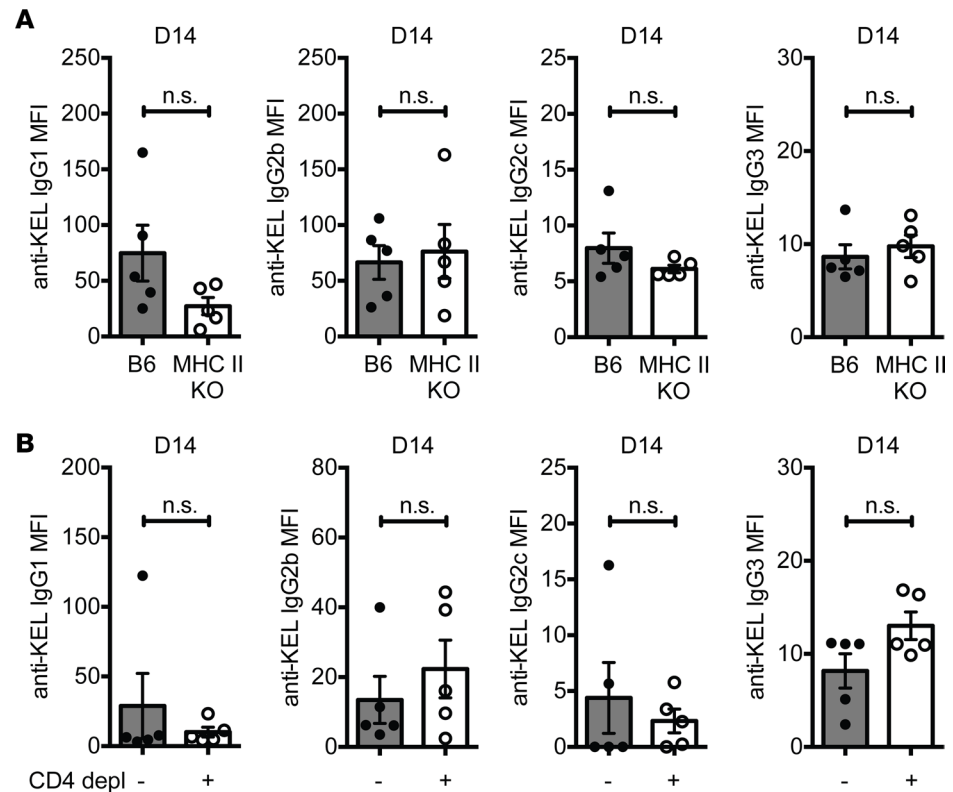


Figure 2. CD4⁺ T cells fail to influence anti-KEL IgG subclass following KEL RBC transfusion. (A) Anti-KEL IgG subtype analysis in the serum of KEL RBC-transfused WT B6 or MHC class II KO (MHC II KO) recipients. **(B)** Anti-KEL IgG subtype analysis in the serum of KEL RBC-transfused WT B6 recipients treated with PBS (-) or monoclonal anti-mouse CD4 depleting antibody (CD4 depl; +). Error bars represent mean \pm SEM. Statistics were generated using an unpaired Mann-Whitney *U* test. There were 5 mice per group. All panels show representative data from experiments reproduced 3 times.

PBS-treated (B6) recipients transfused with KEL RBCs (Supplemental Figure 1B). 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-lymphocytic choriomeningitis virus (anti-LCMV) antibodies (Supplemental Figure 2A), and CD4⁺ T cells were undetectable in the peripheral blood immediately prior to transfusion (Supplemental Figure 2B) and in the spleen of representative recipients evaluated in parallel (Figure 1E).

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 -KO (TCR α KO) recipients, which are genetically deficient in CD4⁺ T cells, were transfused with KEL RBCs (41) (Supplemental Figure 3, A and B). Similar to MHC II KO and CD4⁺ T cell-depleted recipients, there was no statistically significant difference in anti-KEL IgG in TCR α KO and WT B6 recipients following KEL RBC transfusion (Supplemental Figure 3C). 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. Accordingly, sera from recipients depleted or genetically deleted of CD4⁺ T cells were 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 that were statistically similar to those in 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 those in PBS-treated (B6) recipients (Figure 2B). Similar levels of anti-KEL IgG1, IgG2b, IgG2c, and IgG3 were also observed in KEL RBC-transfused TCR α KO recipients compared with WT B6 recipients (Supplemental Figure 3D).

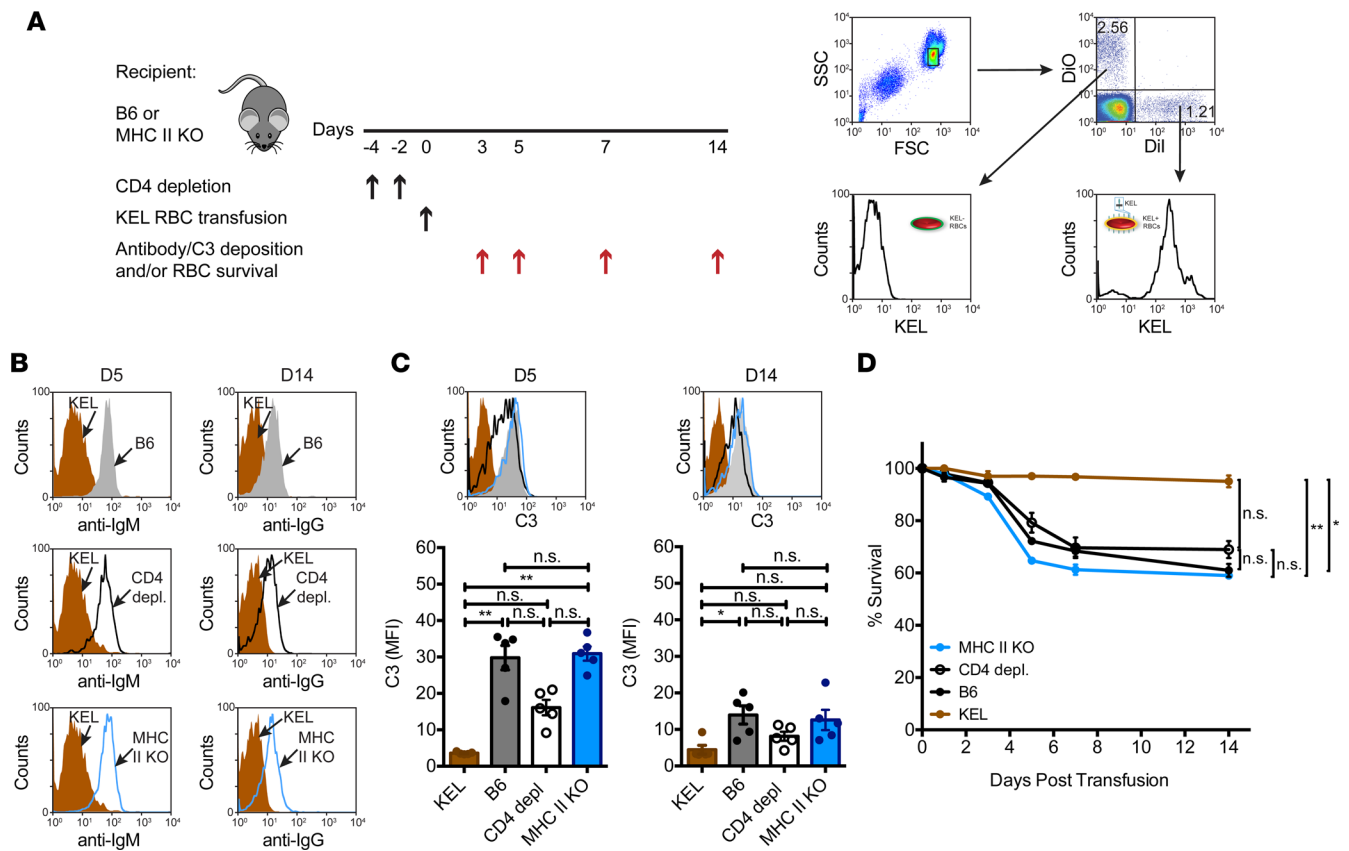


Figure 3. KEL-specific alloantibodies generated in the absence of CD4⁺ T cells bind to transfused KEL RBCs, induce C3 fixation, and facilitate KEL RBC clearance. (A) Schematic of experimental outline. (B) Representative histograms of IgM or IgG binding to KEL-Dii RBCs 5 and 14 days (D5 and D14) after transfusion, respectively. (C) Graphical demonstration with representative histograms of C3 fixation on KEL-Dii RBCs 5 and 14 days after transfusion. (B and C) KEL-positive recipients (brown), PBS-treated B6 recipients (B6; gray), CD4⁺ T cell-depleted recipients (CD4 depl.; black), and MHC class II KO recipients (MHC II KO; blue) are shown. (D) KEL RBC survival 3, 5, 7, and 14 days after transfusion. Error bars represent mean \pm SEM. Statistics were generated using a Kruskal-Wallis with Dunn's multiple-comparisons test in C or a Kruskal-Wallis with Dunn's multiple-comparisons test for day 14 after transfusion in D. There were 5 mice per group. All panels show representative data from experiments reproduced 3 times. * $P < 0.05$; ** $P < 0.01$.

Alloantibodies generated in the absence of CD4⁺ T cells bind to transfused KEL RBCs, 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 an equal mixture of DiI-labeled KEL RBCs and DiO-labeled B6 RBCs to facilitate direct examination of specific changes to KEL RBC survival, as well as antibody binding and complement deposition after transfusion (Figure 3A). KEL-positive recipients transfused with the RBC mixture were additionally included to control for background antibody binding, C3 fixation, and non-immune-mediated clearance of KEL RBCs, as these recipients do not generate anti-KEL antibodies (32).

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, IgM and IgG antibodies bound to transfused KEL RBCs 5 and 14 days after transfusion, respectively, in CD4⁺ T cell-depleted and MHC II KO recipients (Figure 3B). Moreover, C3 fixation was observed in CD4⁺ T cell-depleted and genetically deleted MHC II KO recipients above that of background control KEL-positive recipients transfused with KEL RBCs (Figure 3C). Likewise, anti-KEL alloantibodies formed in the absence of CD4⁺ T cell help induced similar KEL RBC clearance when compared with PBS-treated B6 recipients (Figure 3D). The observed antibody binding, C3 fixation, and RBC clearance were not due to nonspecific effects of transfusing labeled KEL RBCs, as antibody binding, C3 fixation, and KEL RBC clearance were not observed in KEL-positive recipients (Figure 3, B–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 (Table 1).

Table 1. CD4⁺ T cell requirement in anti-KEL antibody formation in the presence or absence of C3, CR1/2, or C5

| | B6 | | C3 KO | | CR1/2 KO | | C5 KO | |
|----------------------------------|----|---|-------|---|----------|---|-------|---|
| CD4 ⁺ T cell-depleted | - | + | - | + | - | + | - | + |
| Anti-KEL IgM | + | + | + | + | + | + | + | + |
| Anti-KEL IgG | + | + | + | - | + | - | + | + |

Recipients deficient in C3 mount an enhanced anti-KEL antibody response. Given that C3 deposition was detectable on transfused KEL RBCs (Figure 3C) and significant evidence suggests a key role for complement in the development of humoral immunity (42–46), 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 (Figure 4A). Unexpectedly, the absence of C3 not only failed to inhibit the development of anti-KEL antibodies following KEL RBC exposure (Figure 4, B and C), but the absence of C3 actually resulted in an enhancement of the anti-KEL antibody response (Figure 4, B and C). Furthermore, while C3 KO recipients transfused with KEL RBCs demonstrated a similar production of anti-KEL IgG1, IgG2b and IgG3 compared to B6 recipients transfused with KEL RBCs, anti-KEL IgG1 and IgG3 in C3 KO recipients appeared to trend toward a higher level when compared to B6 recipients (Figure 4D). The ability of KEL RBCs to induce an enhanced anti-KEL antibody response stands in stark contrast to previous studies demonstrating that optimal IgG formation often requires C3 (47, 48). Moreover, 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. Given the enhanced antibody response to KEL observed in the absence of C3 (Figure 4), and the ability of CD4⁺ T cell-derived cytokines to influence the magnitude and polarization of an immune response (49–54), it is possible that in the absence of C3, CD4⁺ T cells may impact anti-KEL alloantibody formation. To test this, 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 (Figure 5A). 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 5B). However, in contrast to WT 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 5B). The inability to detect anti-KEL IgG in CD4⁺ T cell-depleted C3 KO recipients was not due to sequestration of anti-KEL IgG on transfused KEL RBCs, as KEL RBCs from transfused CD4⁺ T cell-depleted C3 KO recipients lacked detectable IgG binding (Figure 5C). Moreover, while anti-KEL antibodies produced in C3 KO recipients readily cleared KEL RBCs, the absence of detectable anti-KEL IgG in CD4⁺ T cell-depleted C3 KO recipients correlated with a lack of KEL RBC clearance above that of KEL RBCs transfused into control KEL-positive recipients (Figure 5D).

As complement was found to influence anti-KEL antibody formation, we next sought to determine whether complement levels correlate with the anti-KEL antibody response. To test this, we initially determined whether a correlation exists between total C3 levels in the serum and the production of anti-KEL alloantibodies. While C3 levels were variable between recipients, no direct correlation was observed between the level of C3 in a given recipient and the production of anti-KEL IgM or IgG (Figure 6, A and B). As C3 bound to a cell surface is thought to facilitate B cell activation through engagement of CR1/2 (55–58), we next determined whether C3 levels in the serum predict actual C3 fixation on the KEL RBC surface. Similar to the inability of serum C3 to predict anti-KEL IgM and IgG production, serum C3 levels failed to correlate with C3 fixation on the KEL RBC surface (Figure 6C). Anti-KEL antibody engagement is predicted to be responsible for complement fixation on KEL RBCs. As a result, we next examined whether the level of IgM or IgG binding to the KEL RBC surface correlates with C3 fixation. Consistent with this possibility, C3 bound to the cell surface exhibited a strong positive correlation with the degree of IgM and IgG binding to the cell surface (Figure 6, D and E), suggesting that

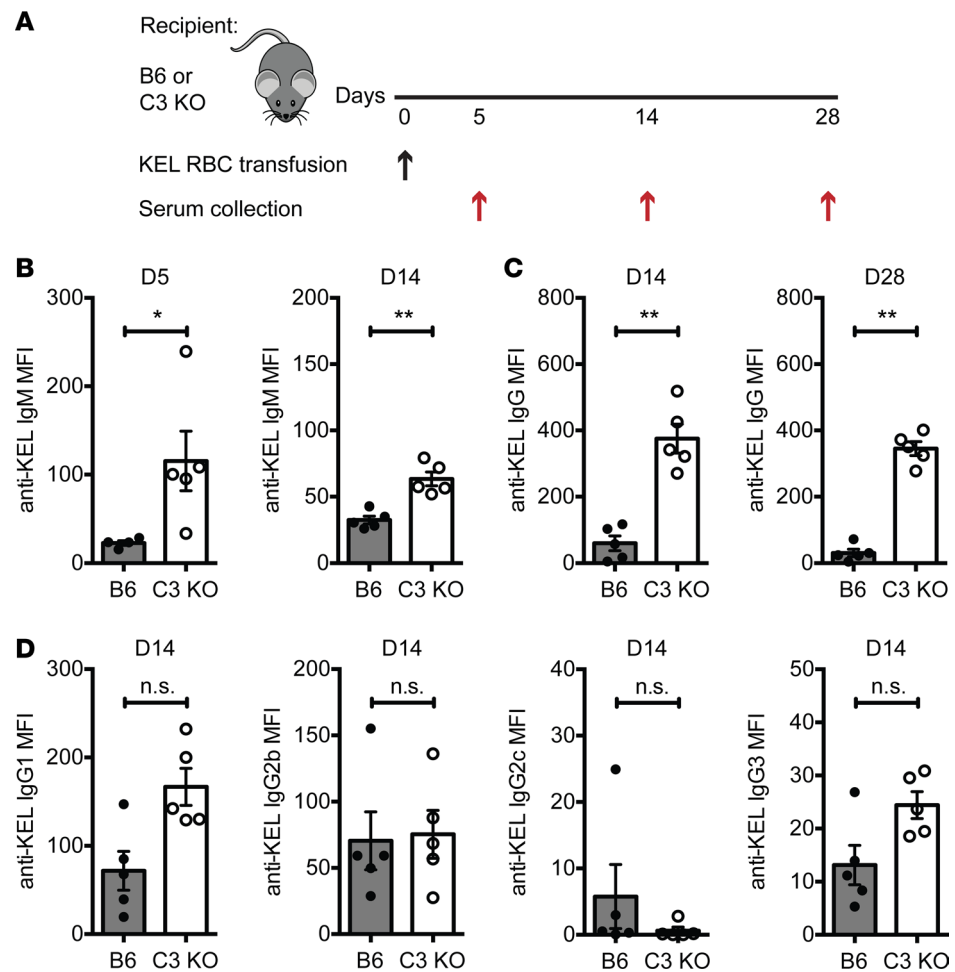


Figure 4. C3 KO recipients generate an enhanced anti-KEL antibody response following KEL RBC transfusion. (A) Schematic of experimental outline. (B and C) Serological examination of (B) anti-KEL IgM on days 5 and 14 (D5 and D14), and (C) anti-KEL IgG on days 14 and 28 after KEL RBC transfusion into WT B6 or C3 KO recipients. (D) Development of anti-KEL IgG1, IgG2b, IgG2c, or IgG3 was examined 14 days after transfusion of KEL RBCs into WT B6 or C3 KO recipients. Error bars represent mean \pm SEM. Statistics were generated using an unpaired Mann-Whitney *U* test. There were 5 mice per group. All panels show representative data from experiments reproduced 3 times. **P* < 0.05; ***P* < 0.01.

antibody engagement is responsible for C3 fixation. In contrast, no correlation existed between C3 levels in the serum and the degree of IgM or IgG detected on KEL RBCs (Figure 6, F and G). Furthermore, no C3 could be detected on KEL RBCs following transfusion into B cell-deficient μ MT mice (Supplemental Figure 4), strongly suggesting that C3 fixation on the KEL RBC surface is antibody dependent as opposed to being a product of the lectin or alternative complement activation pathways. Serum levels of anti-KEL IgM and IgG likewise strongly correlated with bound C3 levels (Figure 6, H and I), with CD4⁺ T cell depletion failing to impact the ability of anti-KEL IgG levels to correlate with bound C3 (Figure 6J). While these results strongly suggest that lower serum C3 levels may not impact the ability of C3 to mediate anti-KEL antibody formation in the absence of CD4⁺ T cells, to more directly test this, we examined alloimmunization to KEL following KEL RBC transfusion in CD4⁺ T cell-depleted C3 heterozygous recipients that possess reduced levels of total serum C3 (Supplemental Figure 5A). Partial deficiency of C3 in C3-heterozygous recipients failed to impact the ability of KEL RBCs to induce anti-KEL IgM and IgG in the presence or absence of CD4⁺ T cells (Supplemental Figure 5B).

C5-deficient recipients induce alloimmunization to KEL independent of CD4⁺ T cell help. As activation of the complement cascade can lead to the downstream generation of C5 (59), and C5 has been shown to play a significant role in CD4⁺ T cell activation, costimulation, as well as survival (60, 61), whether C5 is important in the development of a CD4⁺ T cell-independent anti-KEL antibody response was next investigated. To accomplish this, intact or CD4⁺ T cell-depleted C5 KO recipients were examined for the development of anti-KEL antibodies following KEL RBC transfusion (Figure 7A). Unlike in the absence of C3, exposure

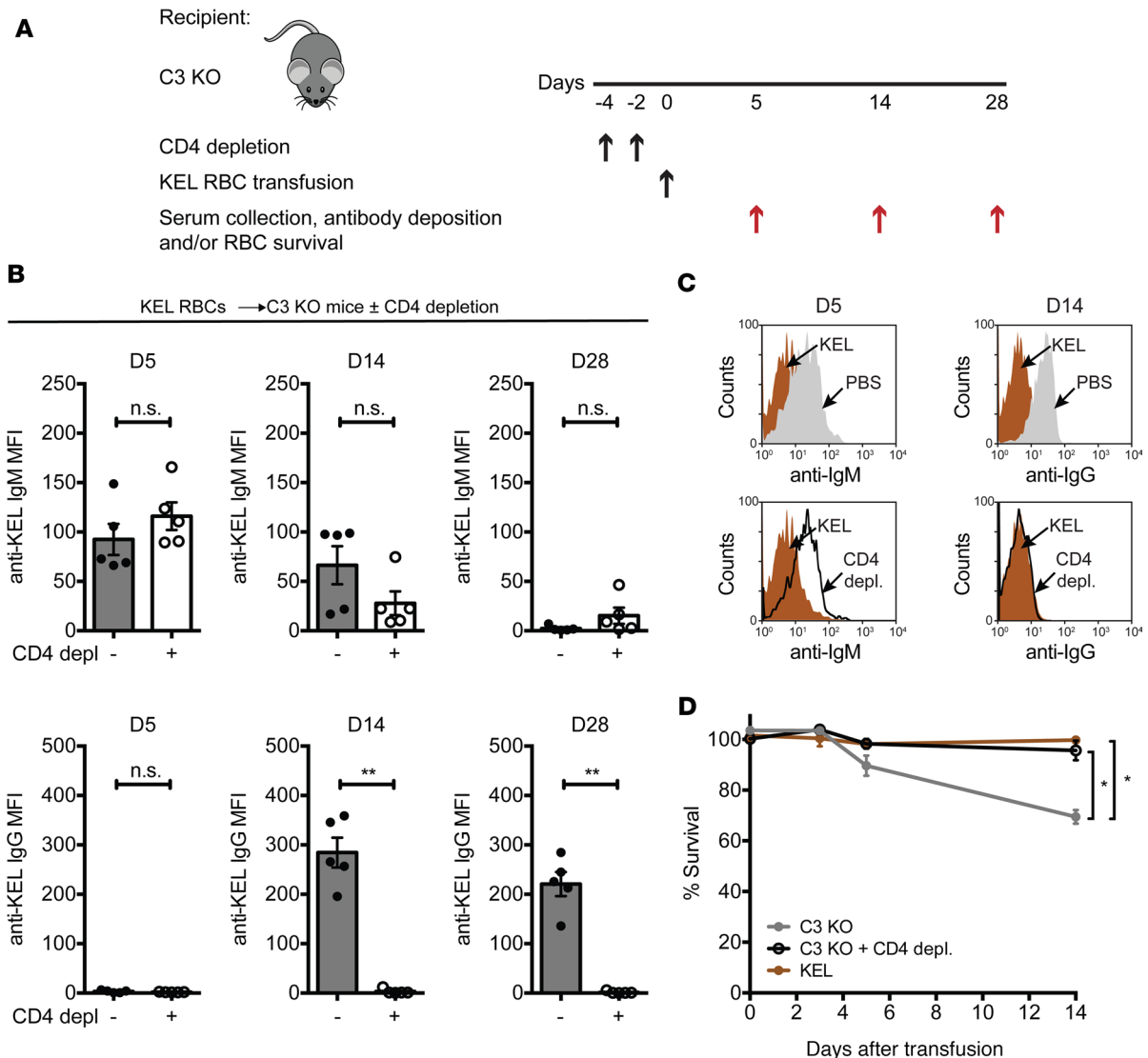


Figure 5. Alloantibody formation to KEL in the absence of C3 is dependent on CD4⁺ T cells. (A) Schematic of experimental outline. (B) C3 KO recipients treated with PBS (-) or monoclonal anti-mouse CD4 depleting antibody (CD4 depl; +) prior to transfusion of KEL RBCs were evaluated for anti-KEL IgM and IgG on 5, 14, and 28 days (D5, D14, and D28) after transfusion. (C) Representative histograms of IgM or IgG binding to KEL RBCs 5 and 14 days after transfusion, respectively, into PBS- (-) or monoclonal anti-CD4 depleting antibody-treated (+) C3 KO recipients. (D) KEL RBC survival 3, 5, and 14 days after transfusion. Error bars represent mean ± SEM. Statistics were generated using an unpaired Mann-Whitney *U* test in B and a Kruskal-Wallis with Dunn's multiple-comparisons test for day 14 after transfusion in D. There were 5 mice per group. All panels show representative data from experiments reproduced 3 times. **P* < 0.05; ***P* < 0.01.

of C5 KO recipients to KEL RBCs in the absence of CD4⁺ T cells resulted in equivalent anti-KEL antibody formation (Figure 7B); the antibody response to KEL RBCs in C5 KO recipients both in the presence and absence of CD4⁺ T cells was functionally similar, as anti-KEL antibodies bound to transfused KEL RBCs, induced C3 fixation, and facilitated KEL RBC clearance (Figure 7, C–E).

Anti-KEL antibody formation in recipients deficient in CR1/2 is dependent on CD4⁺ T cells. Given that complement receptor ligation with CD19 and the B cell receptor can act as a costimulatory signal for B cells (55–58) and C3 KO recipients were unable to mount a detectable anti-KEL IgG response in the absence of CD4⁺ T cells (Figure 5B), 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, WT B6 and CR1/2 KO recipients were transfused with KEL RBCs and subsequently tested for the development of antibodies against KEL (Supplemental Figure 6A). Exposure of CR1/2 KO recipients to KEL RBCs resulted in an anti-KEL IgM response that was delayed but similar to that in B6 WT recipients (Supplemental Figure 6B).

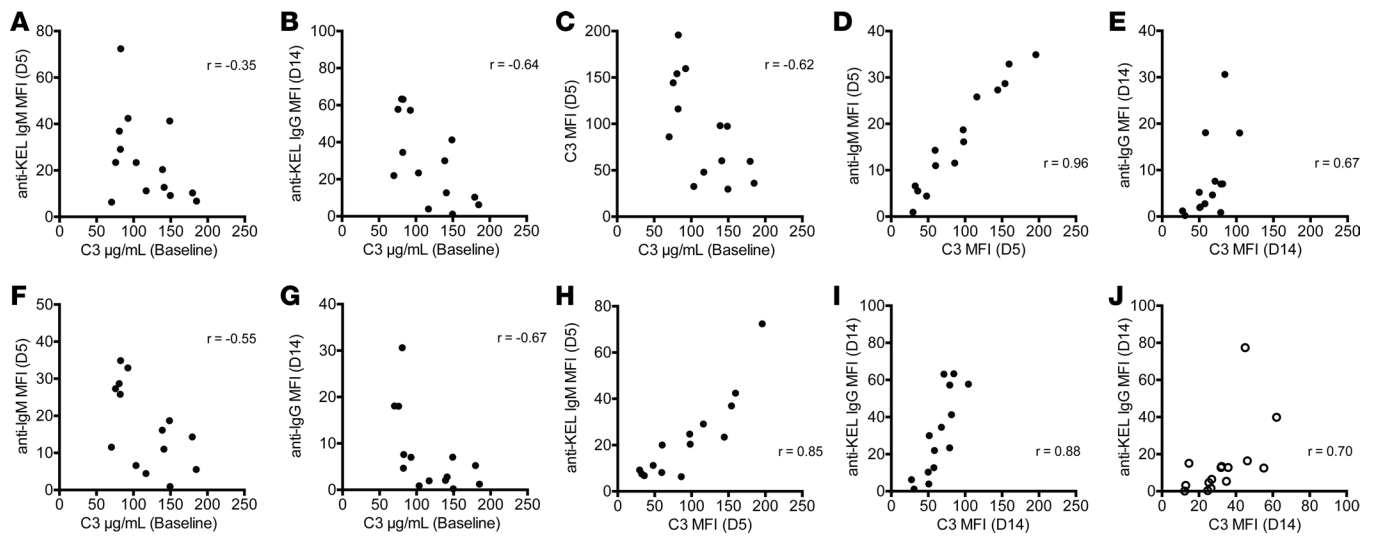


Figure 6. C3 fixation levels correlate with anti-KEL antibody formation. (A and B) Baseline serum C3 levels were correlated to (A) anti-KEL IgM or (B) anti-KEL IgG in serum at 5 or 14 days (D5 or D14) after transfusion into WT B6 recipients. (C) Correlation between baseline serum C3 levels and C3 fixation on transfused KEL RBCs 5 days after transfusion. (D and E) Level of C3 fixation on transfused KEL RBCs correlated to (D) IgM or (E) IgG bound to transfused KEL RBCs 5 and 14 days after transfusion, respectively. (F and G) Correlation between baseline serum C3 levels and (F) IgM or (G) IgG binding to transfused KEL RBCs 5 and 14 days after transfusion, respectively. (H and I) Levels of C3 fixed on transfused KEL RBCs were correlated to (H) anti-KEL IgM or (I) anti-KEL IgG in serum at 5 and 14 days after transfusion, respectively. (J) Levels of C3 fixed on transfused KEL RBCs were correlated to anti-KEL IgG in serum at 14 days after transfusion into CD4⁺ T cell-depleted recipients. Statistics were generated using Spearman's correlation test in all panels. There were 15 mice in each experimental group. All panels are representative of data from experiments reproduced 3 times. Positive correlations were observed for D ($P < 0.0001$), E ($P < 0.05$), H ($P < 0.001$), I ($P < 0.001$), and J ($P < 0.05$).

However, consistent with the critical role of CR1/2 in B cell responses in general (45, 62), transfusion of CR1/2 KO recipients with KEL RBCs resulted in a depressed anti-KEL IgG response compared with WT B6 recipients (Supplemental Figure 6C). Taken together, these results suggest that similar to the immune response to other antigens (43–45, 62–67), CR1/2 expression is important in facilitating the development of anti-KEL IgG antibodies.

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 (Figure 8A). 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 8B). However, in the absence of CD4⁺ T cells, CR1/2 KO recipients failed to generate a detectable anti-KEL IgG response compared with PBS-treated CR1/2 KO recipients (Figure 8B). The inability to detect anti-KEL IgG in CD4⁺ T cell-depleted CR1/2 KO recipients was not due to sequestration of anti-KEL IgG on transfused KEL RBCs, as KEL RBCs from transfused CD4⁺ T cell-depleted CR1/2 KO recipients lacked detectable IgG binding (Figure 8C). Furthermore, the inability of CR1/2 KO recipients to generate anti-KEL IgG did not appear to be due to lack of C3 fixation on the KEL RBC surface, as C3 could be readily detected on the KEL RBC surface (Figure 8D). While anti-KEL antibodies appeared to also induce the removal of KEL RBCs, the absence of detectable anti-KEL IgG in CD4⁺ T cell-depleted CR1/2 KO recipients correlated with reduced KEL RBC clearance (Figure 8E). 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 (Table 1). 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. As our data indicate that C3-CR1/2 ligation may facilitate the development of a T cell-independent antibody response to transfused KEL RBCs in WT B6 recipients, and previous studies demonstrated that CR1/2 is only expressed by B cells and follicular DCs (FDCs) in mice (44, 45, 48, 63), we investigated whether CR1/2 expression on hematopoietic-derived B cells or non-hematopoietic-derived

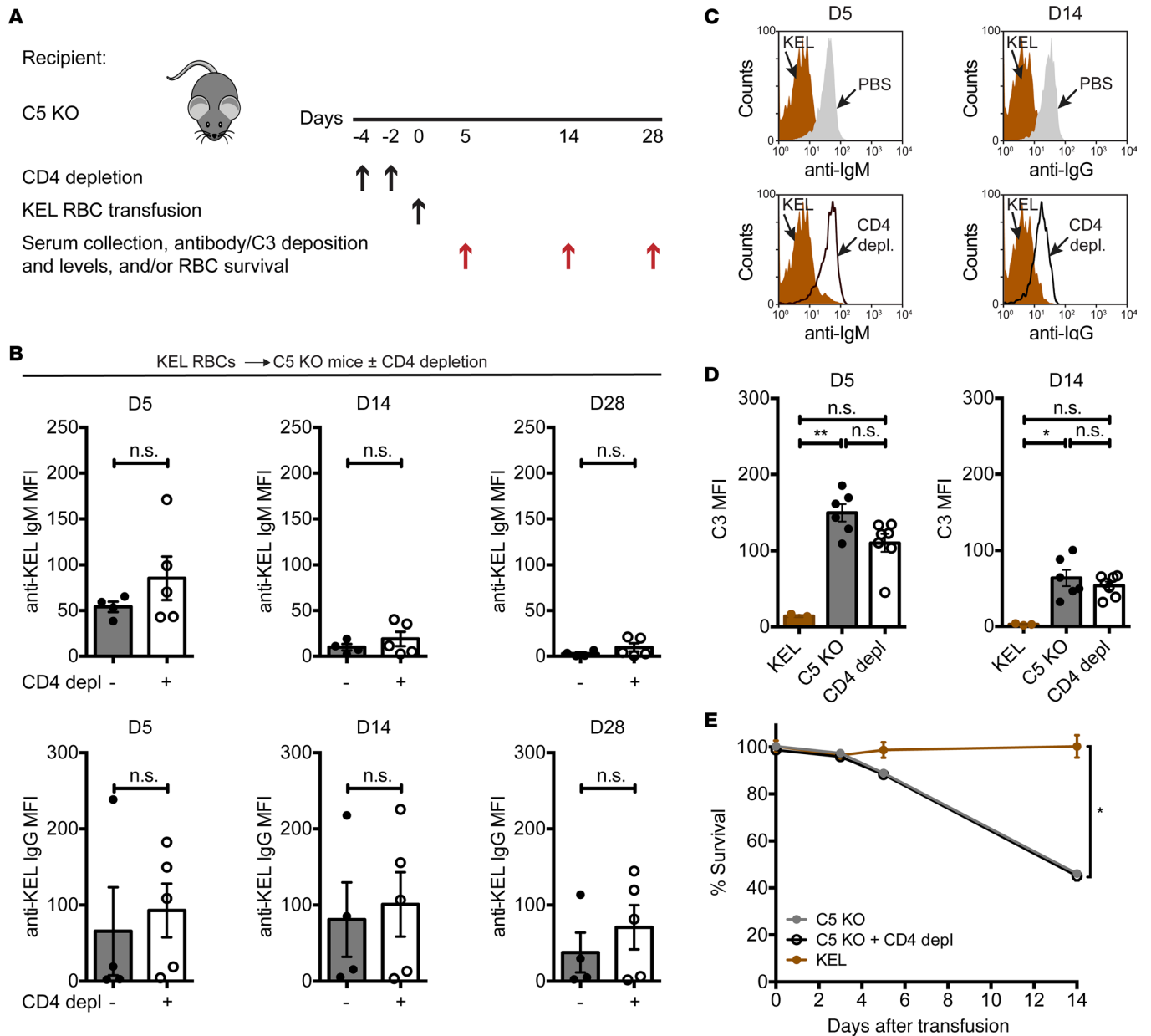


Figure 7. Anti-KEL antibodies formed in the absence of C5 do not require CD4⁺ T cells. (A) Schematic of experimental outline. (B) Serological analysis of anti-KEL IgM and IgG was evaluated 5, 14, and 28 (D5, D14, and D28) days after transfusion of KEL RBCs into PBS- (-) or monoclonal anti-CD4 depleting antibody-treated (CD4 depl; +) C5 KO recipients. (C) Representative histograms of IgM or IgG on transfused KEL-DII RBCs examined 5 and 14 days after transfusion, respectively. (D) C3 fixation on transfused KEL RBCs was evaluated 5 and 14 days after transfusion. (E) KEL RBC survival 3, 5, and 14 days after transfusion. Error bars represent mean ± SEM. Statistics were generated using an unpaired Mann Whitney U test in B, a Kruskal-Wallis with Dunn's multiple-comparisons test in D, and a Kruskal-Wallis with Dunn's multiple-comparisons test for day 14 after transfusion in E. There were either 5 (B) or 3–7 (C–E) mice per group. All panels show representative data from experiments reproduced 2 (C–G) or 3 (B) times. **P* < 0.05; ***P* < 0.01.

FDCs is required for the development of an anti-KEL alloantibody response in the absence of CD4⁺ T cells. To test this, CR1/2 KO recipients were lethally irradiated and reconstituted with B6 bone marrow to generate recipients deficient in CR1/2 specifically on non-hematopoietic cells. Following reconstitution, recipients were depleted of CD4⁺ T cells and subsequently transfused with KEL RBCs (Figure 9A).

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 9B). 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

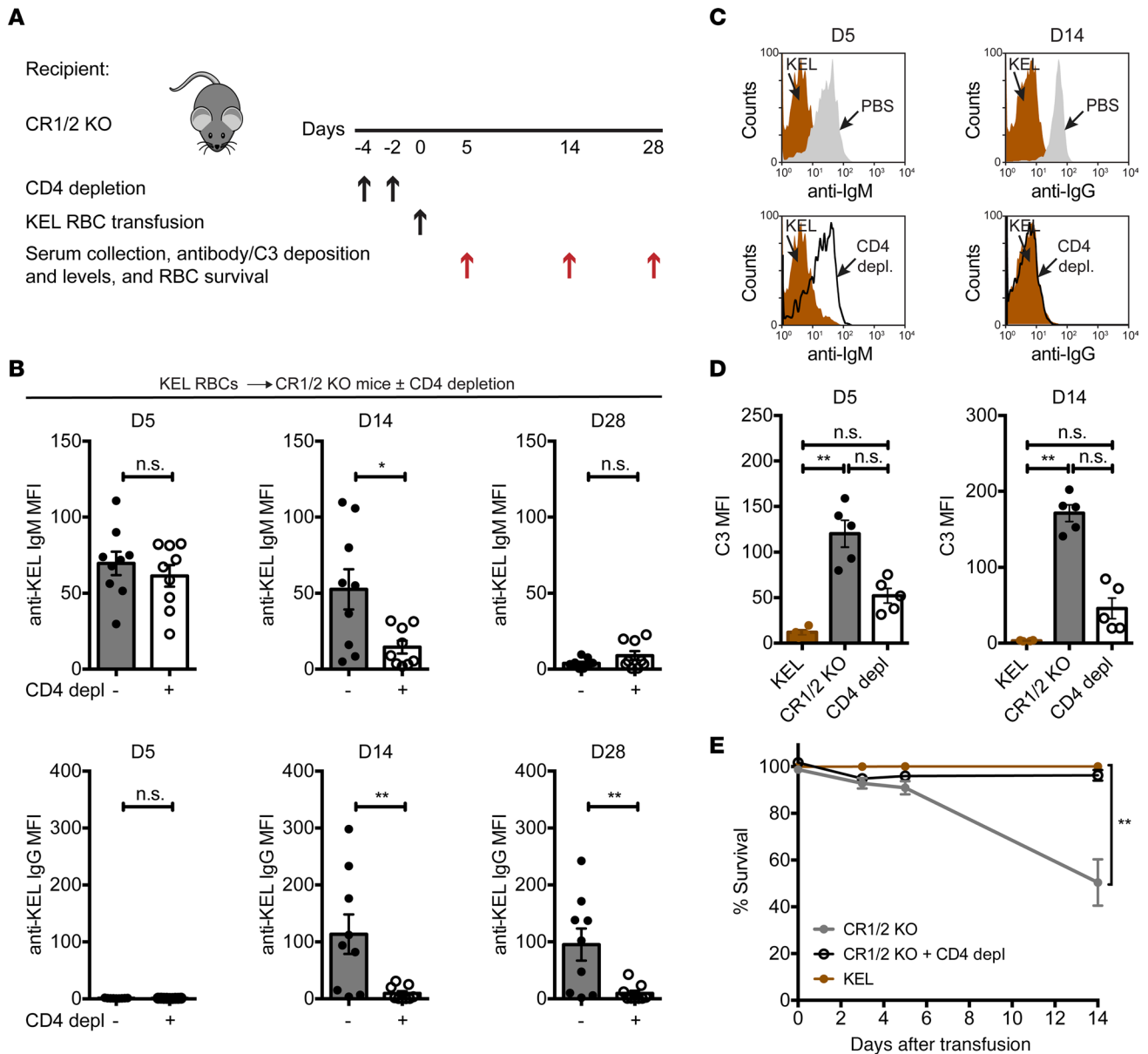


Figure 8. Depletion of CD4⁺ T cells in CR1/2 KO recipients does not lead to a significant change in anti-KEL IgG following KEL RBC transfusion. (A) Schematic of experimental outline. (B) Serological analysis of anti-KEL IgM and IgG was evaluated on days 5, 14, and 28 (D5, D14, and D28) after transfusion of KEL RBCs into PBS- (-) or monoclonal anti-mouse CD4 depleting antibody-treated (CD4 depl; +) CR1/2 KO recipients. (C) Representative histograms of IgM or IgG on transfused KEL RBCs examined 5 and 14 days after transfusion, respectively. (D) C3 fixation on transfused KEL RBCs was evaluated 5 and 14 days after transfusion. (E) KEL RBC survival 3, 5, and 14 days after transfusion. Error bars represent mean ± SEM. Statistics were generated using an unpaired Mann Whitney *U* test in B, a Kruskal-Wallis with Dunn’s multiple-comparisons test in D, and a Kruskal-Wallis with Dunn’s multiple-comparisons test for day 14 after transfusion in E. There were either 9 (B) or 5 mice per group (C–E). All panels show representative data from experiments reproduced 2 times. **P* < 0.05; ***P* < 0.01.

response following KEL RBC transfusion (Figure 9B). 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, the 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 to generate hematopoietic cells specifically deficient in CR1/2. Following engraftment, recipients were depleted of CD4⁺ T cells and subsequently transfused with KEL RBCs (Figure 9C). 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 9D). These results indicate that non-hematopoietic expression

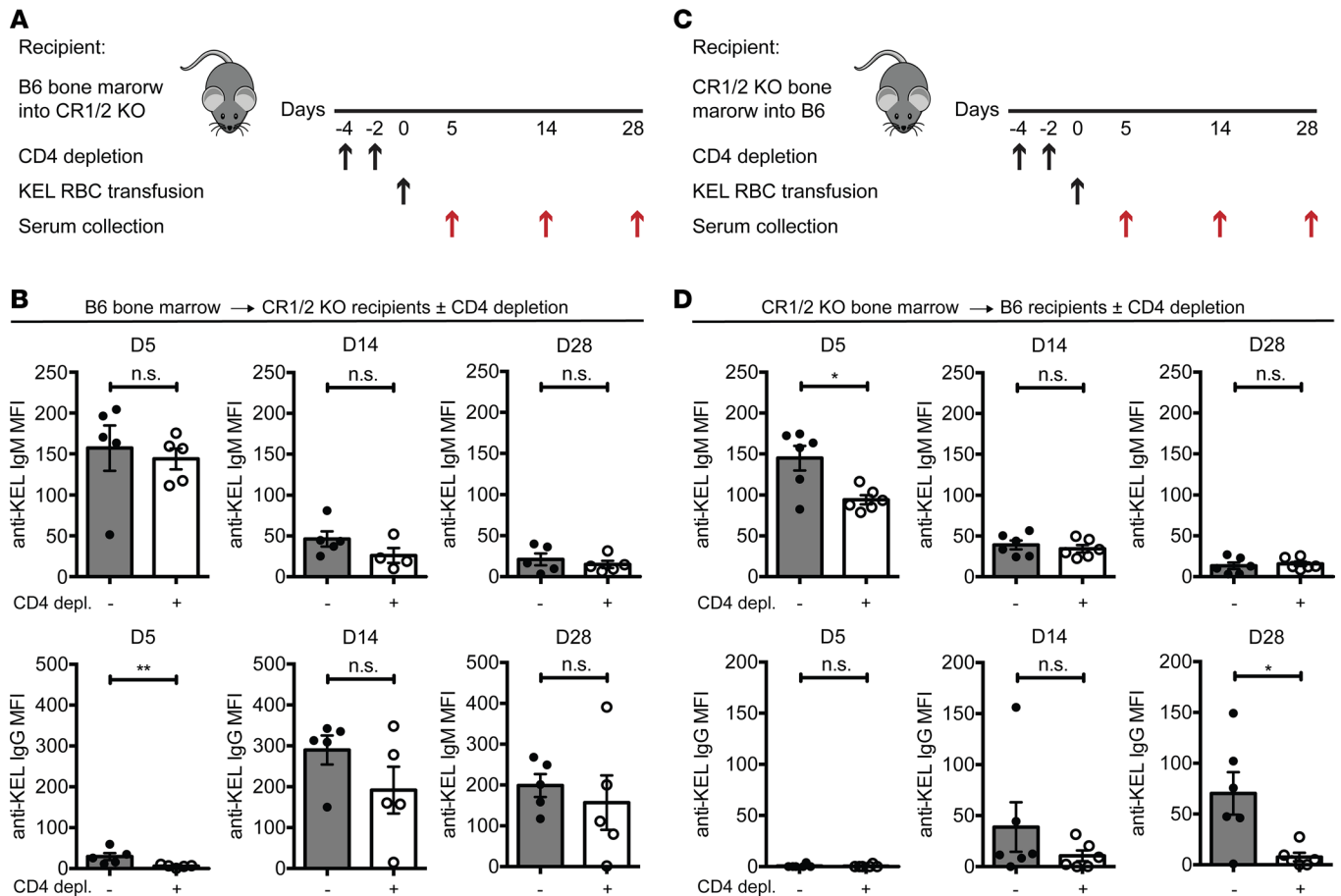


Figure 9. Hematopoietic expression of CR1/2 rescues development of anti-KEL IgG in the absence of CD4⁺ T cells. (A) Schematic of experimental outline for B. (B) Serological analysis of anti-KEL IgM and IgG development was evaluated on days 5, 14, and 28 (D5, D14, and D28) after transfusion into PBS- (-) or a monoclonal anti-CD4 depleting antibody-treated (CD4 depl; +) CR1/2 KO recipients transplanted with B6 bone marrow. (C) Schematic of experimental outline for D. (D) Serological analysis of anti-KEL IgM and IgG development was evaluated on days 5, 14, and 28 after transfusion into PBS- (-) or monoclonal anti-CD4 depleting antibody-treated (+) B6 recipients transplanted with CR1/2 KO bone marrow. Error bars represent mean \pm SEM. Statistics were generated using an unpaired Mann Whitney *U* test. There were either 5 (B) or 6 mice per group (D). All panels show representative data from experiments reproduced 2 times. **P* < 0.05; ***P* < 0.01.

of CR1/2 alone does not induce a CD4⁺ T cell-independent antibody response to the KEL antigen on transfused KEL RBCs. Rather, the 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 (Table 2).

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 (13, 14, 20), indicating a requirement for CD4⁺ T cell help. Moreover, recent studies have demonstrated that RhD (14), Jk^k (68), and Kell-derived peptides (69) can be presented by distinct HLA variants to T lymphocytes (18, 69). 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 independently of C3 and CR1/2. However, unexpectedly, C3 and CR1/2 were found to regulate whether humoral immunity to KEL occurred independently 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

Table 2. Dependence of CD4⁺ T cells on anti-KEL antibody formation in the presence of CR1/2 expression on hematopoietic or non-hematopoietic cells

| | B6 BM × CR1/2 KO recipient | | CR1/2 KO BM × B6 recipient | |
|----------------------------------|----------------------------|---|----------------------------|---|
| CD4 ⁺ T cell-depleted | - | + | - | + |
| Anti-KEL IgM | + | + | + | + |
| Anti-KEL IgG | + | + | + | - |

not exclude the possibility that when C3 is present KEL can activate CD4⁺ T cells and that these CD4⁺ T cells may influence anti-KEL alloantibody production following KEL RBC exposure. 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 (e.g., LPS) that can elicit nonspecific or polyclonal activation of B cells via pattern recognition receptor (e.g., Toll like receptors) ligation are traditionally classified as T cell-independent type I antigens (70). 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 (e.g., 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 (71). 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 CR1/2 ligation of C3 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 (42, 43, 72). However, in contrast to this classical paradigm, wherein antigens are thought to be intrinsically 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 such as 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 (48, 55, 56, 58, 73–75), 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 (69) — which therefore suggests 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 possess the ability 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 that CD4⁺ T cells are required to induce an alloantibody response to Kell. While several RBC alloantigens appear to be associated with particular HLA alleles (13–22), implicating a potential requirement for CD4⁺ T cells in alloantibody formation, alloimmunization to other RBC antigens fails to demonstrate a similar association with HLA type (76), suggesting that CD4⁺ T cell-independent antibody responses may also occur following exposure to other RBC alloantigens.

One advantage of a model system like KEL is that it provides the opportunity to directly examine the contribution of immunologic factors (e.g., CD4⁺ T cells and C3) that may govern the alloantibody response to KEL; elucidation of such immunologic pathways would not be ethically feasible or logistically possible to study in a detailed fashion in humans. This in part is due to the fact that while RBC alloimmunization occurs clinically as a consequence of therapeutic RBC transfusion, intentionally inducing RBC alloimmunization to antigens besides 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. Importantly, alloantibodies formed to the KEL antigen in this model in the presence or absence of CD4⁺ T cells recapitulated key features of clinical RBC alloimmunization, as anti-KEL antibodies possessed the ability to clear KEL RBCs. Previous studies using the HOD system indeed demonstrated that CD4⁺ T cells are required for anti-HOD IgG formation, suggesting that the ability to drive alloantibodies independent of CD4⁺ T cells may be somewhat unique to the KEL antigen. However, antibodies generated following HOD RBC transfusion are primarily directed against the non-blood group antigen HEL portion of the chimeric HOD (HEL, OVA, and Duffy) antigen, making it difficult to determine whether the HOD RBC alloantibody response reflects other clinically relevant RBC alloantigens. As a result, additional RBC alloimmunization models centered on other RBC alloantigens, such as Kidd and S/s/U, are needed in order to determine whether other RBC alloantigens likewise possess the ability to drive a C3-dependent, CD4⁺ T cell-independent alloantibody response.

The potential ability of an RBC alloantigen to induce antibodies through a CD4⁺ T cell-independent but C3-dependent pathway is important, as CD4⁺ T cells have been postulated as a possible target in the prevention of RBC alloimmunization. However, as previously noted, whether these findings recapitulate clinical alloantibody formation to KEL or other RBC alloantigens, or simply provide a model to study C3-dependent, CD4⁺ T cell-independent-mediated alloimmunization remains unclear. Furthermore, while we failed to detect any changes in the quantity or quality of the antibody response to KEL in the presence or absence of CD4⁺ T cells, these results certainly do not rule out that when present, CD4⁺ T cells may influence the antibody response in ways that are simply not detected in this model system. However, these findings do suggest that therapies targeted toward CD4⁺ T cells alone may not be sufficient to regulate other undesirable immune responses toward some RBC alloantigens. As similar immune responses may occur in certain autoimmune diseases, it is also 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 (77–83).

While a variety of traditional model antigens, including hen egg lysozyme, ovalbumin, NP, and key-hole 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 (45, 84–86), 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 such as 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 insights into a role for complement in the development of humoral immunity.

The lack of correlation between C3 levels in the serum and anti-KEL antibody formation strongly suggests that anti-KEL IgM formed following KEL RBC exposure may engage the KEL antigen and subsequently induce C3 fixation on the surface of KEL RBCs. Consistent with this, no antibody or C3 could be detected on KEL RBCs following transfusion into B cell-deficient μ MT recipients, ruling out the possibility that the lectin

or alternative complement pathways drive C3 fixation following KEL RBC exposure. The possibility that C3 directly engages CR1/2 is consistent with previous observations and suggests that direct C3 binding to CR1/2, as opposed to downstream complement players such as C5, likely drives CD4⁺ T cell-independent antibody formation to the KEL antigen (55–58). Indeed, C5 KO recipients continued to form anti-KEL IgG independently of CD4⁺ T cells. As variable levels of anti-KEL IgM develop following transfusion of KEL RBCs into C3 KO and WT B6 recipients, the initial level of anti-KEL antibody formation appears to occur independently of C3. However, once IgM engages KEL RBCs and fixes complement, C3 may engage CR1/2, possibly facilitating the development of anti-KEL IgG. While it is difficult to discern whether correlations between complement levels on the cell surface and IgG antibody formation reflect antibody-mediated complement deposition, C3-mediated antibody formation and subsequent antibody binding, or both, the requirement of CR1/2 for CD4⁺ T cell-independent antibody formation strongly suggests that C3 engagement of CR1/2 directly facilitates anti-KEL IgG formation and that the level of C3 may therefore influence the magnitude of the anti-KEL IgG response.

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 complement may be an important innate 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 were in a murine model, testing the hypothesis in a human setting would be required before any clinical conclusions can be drawn. Nonetheless, the current findings are relevant to human medicine in that they suggest an underappreciated immunological modulator of RBC alloimmunization.

Methods

Mice. Female B6 (CD45.2 C57BL/6; H-2^b) and CD45.1 B6 (B6.SJL-PtprcaPepcb/BoyCrCr1) mice were purchased from the National Cancer Institute or Charles River. μ MT (B6.129S2-*Ighm*^{m1Cgn}/J; H-2^b), MHC II KO (B6.129S2-*H2*^{dAb1-Ea}/J; H-2^b), TCR α KO (B6.129S2-*Tcr α* ^{m1Mon}/J; H-2^b), C5 KO (B6N(Cg)-*Hc*^{m1b(EUCOMM)Wsi}/J; H-2^b) and C3 KO (B6.129S4-*C3*^{m1Cr}/J; H-2^b) mice were purchased from the Jackson Laboratory at 6–8 weeks of age. CR1/2-deficient (CR1/2 KO) mice were a gift from V. Michael Holers (University of Colorado, Aurora, Colorado, USA). All mice were on a B6 background and used at 8–12 weeks of age. Transgenic KEL (H-2^b) donors were a gift from James C. Zimring (Bloodworks Northwest, Seattle, Washington, USA) (30). C3-heterozygous mice recipients were a gift from David Archer (Emory University). Mice were bred and housed in Emory University Department of Animal Resources facilities, and all procedures were performed according to IACUC-approved protocols.

Antibodies for flow cytometry. APC anti-mouse CD4 (clone RM4-5), FITC anti-mouse CD3 ϵ (clone: 145-2C11), PE anti-mouse CD8 α (clone 53-6.7), and APC streptavidin were bought from BD Biosciences. Biotinylated polyclonal anti-mouse C3 was obtained from Cedarlane, and APC polyclonal goat anti-mouse IgG, FITC polyclonal goat anti-mouse IgM, PE polyclonal goat anti-mouse IgG1, PE polyclonal anti-mouse IgG2c, PerCP polyclonal goat anti-mouse IgG3, Alexa Flour 488 polyclonal goat anti-mouse IgG2b, and polyclonal goat anti-mouse IgG HRP were purchased from Jackson ImmunoResearch Laboratories Inc.

Cellular depletion and RBC transfusion. CD4⁺ T cell depletion was achieved by 2 intraperitoneal injections of 250 μ g monoclonal anti-mouse CD4 depleting antibody (clone: GK1.5; Bio X Cell) 4 and 2 days prior to transfusion (87). Efficacy of CD4⁺ T cell depletion was assessed by staining peripheral blood (prior to transfusion) or splenocytes with FITC anti-mouse CD3 ϵ + PE anti-mouse CD8 α + APC anti-mouse CD4 (clone: RM-45). Samples were run on a 4-color BD FACSCalibur and analyzed using FlowJo. Donor KEL whole blood was collected at 1:8 into acid citrate dextrose (ACD; Vacutainer) and washed 3 times with 1 \times DPBS. Depleted recipients were then transfused via the lateral tail vein with 50 μ l packed KEL RBCs diluted in 1 \times DPBS to a 300 μ l 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 (88). Briefly, B6 recipients treated with PBS or monoclonal anti-mouse CD4 depleting antibody (clone: GK1.5) were infected with 2 \times 10⁶ PFU LCMV (clone 13). Serum collected 15 days after infection was 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 1:5,000 in blocking buffer.

Survival of RBCs, serum C3 as well as antibody binding and C3 fixation on transfused RBCs in vivo. WT B6 or transgenic KEL whole blood was collected 1:8 into ACD and washed 3 times with $1\times$ DPBS. Resulting B6 packed RBCs were labeled with 3,3'-dihexadecyloxycarbocyanine perchlorate (DiO; Molecular Probes), while KEL-packed RBCs were labeled with chloromethylbenzamido 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI; Molecular Probes), as previously described (33). Briefly, 1 ml packed RBCs was diluted 1:10 in $1\times$ 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 3 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 $50\ \mu\text{l}$ of each type of blood diluted in $1\times$ DPBS to a total volume of $300\ \mu\text{l}$. Serum C3 levels was examined on day 0 at baseline using a C3 ELISA kit (Abcam). At the time points indicated after transfusion, KEL RBC survival was examined by comparing the survival of transfused KEL-DiI RBCs normalized to the tracer B6-DiO RBCs. Antibody binding and C3 fixation were examined on days 5 and 14 after transfusion. Antibody binding was evaluated by incubating samples for 30 minutes at 4°C with APC anti-mouse IgM or APC anti-mouse IgG diluted 1:100 in $1\times$ DPBS + 2% BSA buffer. C3 fixation was assessed by incubating samples for 30 minutes at 4°C with biotinylated anti-mouse C3 antibody diluted 1:100 in $1\times$ DPBS + 2% BSA buffer, followed by APC streptavidin diluted 1:100 in $1\times$ 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 (MFI) was used to measure antibody binding and C3 fixation on transfused KEL-DiI RBCs.

Seroanalysis. Serum collected 5, 14, and 28 days after transfusion was evaluated for anti-KEL antibodies by indirect immunofluorescence staining, as previously described (33). 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. As neat serum demonstrated maximum antibody detection by our indirect immunofluorescence staining assay (Supplemental Figure 1), neat serum was employed to evaluate anti-KEL antibody formation. After 15 minutes, samples were washed 3 times with FACS buffer ($1\times$ DPBS + 2% BSA + 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. For all samples, serological examination occurred through indirect immunofluorescence staining using KEL and B6 RBCs, and neat serum. The MFI values were determined by subtracting background MFI values obtained following incubation with B6 RBCs from values observed following incubation with KEL RBCs. Samples were run on a 4-color BD FACSCalibur and analyzed using FlowJo; MFI 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, 31, 33, 89). 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 2 doses of 550 Gy 3 hours apart using a gamma irradiator. Twenty-four hours later, bone marrow was harvested from CD45.2 B6, CD45.1 B6, and/or CR1/2 KO donors, as previously described (90). 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 $70\ \mu\text{m}$ filter to remove debris. Marrow was then centrifuged for 10 minutes at $300\ g$ and washed 2 times with $1\times$ PBS. Cells were counted using a hemocytometer and reconstituted in $1\times$ PBS to 10×10^6 cells/ml. $500\ \mu\text{l}$ of the bone marrow was then transfused into indicated recipients via the lateral tail vein (5×10^6 bone marrow cells total). After transplantation, recipients were treated with 1 mg/ml neomycin sulfate (MilliporeSigma) in sterile drinking water that was changed weekly. Following engraftment, mice were depleted of CD4^+ T cells 4 and 2 days prior to transfusion. All recipients were transfused with $50\ \mu\text{l}$ packed KEL RBCs in $300\ \mu\text{l}$ of $1\times$ PBS by lateral tail vein injection.

Statistics. Statistical analysis was performed using an unpaired parametric and nonparametric *t* test, unpaired Mann Whitney *U* test, Kruskal-Wallis with Dunn's multiple-comparison test, or Spearman's correlation test. Significance was determined by a *P* value less than 0.05.

Author contributions

SRP, AM, and SRS designed the research study. SRP and AM carried out and analyzed experiments together with SC, CMA, CLM, RPJ, MS, JL, KGP, AB, and NHS. AW, PEZ, and JEH provided critical support. SRP and SRS wrote the manuscript, which was additionally edited and commented on by the others.

Acknowledgments

This work was supported in part by grants from the Burroughs Wellcome Trust Career Award for Medical Scientists, NIH grants DP5OD019892, HL135575, and HL132819 to SRS and 1F31HL131428 to AM. The authors would like to thank Carl Davis, Anna Kamphorst, and George Punkosdy for experimental feedback and guidance. The authors would also like to thank the Winship Biostatistics and Bioinformatics Core for statistical guidance

Address correspondence to: Sean R. Stowell, Center for Transfusion Medicine and Cellular Therapies, Department of Laboratory Medicine and Pathology, 615 Michael Street, Atlanta, Georgia 30322, USA. Phone: 404.727.8538; Email: srstowe@emory.edu.

- Vichinsky EP, Earles A, Johnson RA, Hoag MS, Williams A, Lubin B. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. *N Engl J Med*. 1990;322(23):1617–1621.
- Yazdanbakhsh K, Ware RE, Noizat-Pirenne F. Red blood cell alloimmunization in sickle cell disease: pathophysiology, risk factors, and transfusion management. *Blood*. 2012;120(3):528–537.
- Ambruso DR, et al. Experience with donors matched for minor blood group antigens in patients with sickle cell anemia who are receiving chronic transfusion therapy. *Transfusion*. 1987;27(1):94–98.
- Rosse WF, et al. Transfusion and alloimmunization in sickle cell disease. The cooperative study of sickle cell disease. *Blood*. 1990;76(7):1431–1437.
- Hillyer CD, Shaz BH, Winkler AM, Reid M. Integrating molecular technologies for red blood cell typing and compatibility testing into blood centers and transfusion services. *Transfus Med Rev*. 2008;22(2):117–132.
- Nickel RS, et al. Impact of red blood cell alloimmunization on sickle cell disease mortality: a case series. *Transfusion*. 2016;56(1):107–114.
- Stowell SR, et al. Alloantibodies to a paternally derived RBC KEL antigen lead to hemolytic disease of the fetus/newborn in a murine model. *Blood*. 2013;122(8):1494–1504.
- Moise KJ. Fetal anemia due to non-rhesus-D red-cell alloimmunization. *Semin Fetal Neonatal Med*. 2008;13(4):207–214.
- Telen MJ, Afenyi-Annan A, Garrett ME, Combs MR, Orringer EP, Ashley-Koch AE. Alloimmunization in sickle cell disease: changing antibody specificities and association with chronic pain and decreased survival. *Transfusion*. 2015;55(6 Pt 2):1378–1387.
- Chonat S, et al. Contribution of alternative complement pathway to delayed hemolytic transfusion reaction in sickle cell disease. *Haematologica*. 2018;103(10):e483–e485.
- Chou ST, Jackson T, Vege S, Smith-Whitley K, Friedman DF, Westhoff CM. High prevalence of red blood cell alloimmunization in sickle cell disease despite transfusion from Rh-matched minority donors. *Blood*. 2013;122(6):1062–1071.
- Pourazar A. Red cell antigens: Structure and function. *Asian J Transfus Sci*. 2007;1(1):24–32.
- Chiaroni J, et al. HLA-DRB1 polymorphism is associated with Kell immunisation. *Br J Haematol*. 2006;132(3):374–378.
- Stott LM, Barker RN, Urbaniak SJ. Identification of alloreactive T-cell epitopes on the Rhesus D protein. *Blood*. 2000;96(13):4011–4019.
- Baleotti W, Ruiz MO, Fabron A, Castilho L, Giuliani S, Donadi EA. HLA-DRB1*07:01 allele is primarily associated with the Diego a alloimmunization in a Brazilian population. *Transfusion*. 2014;54(10):2468–2476.
- Chu CC, et al. Anti-“Mi(a)” immunization is associated with HLA-DRB1*0901. *Transfusion*. 2009;49(3):472–478.
- Maluskova A, et al. Association of HLA-DRB1 and HLA-DQB1 with red-blood-cell alloimmunization in the Czech population. *Vox Sang*. 2017;112(2):156–162.
- Noizat-Pirenne F, et al. Relative immunogenicity of Fya and K antigens in a Caucasian population, based on HLA class II restriction analysis. *Transfusion*. 2006;46(8):1328–1333.
- Picard C, et al. Positive association of DRB1 04 and DRB1 15 alleles with Fya immunization in a Southern European population. *Transfusion*. 2009;49(11):2412–2417.
- Reviron D, et al. HLA-DRB1 alleles and Jk(a) immunization. *Transfusion*. 2005;45(6):956–959.
- Schonewille H, Doxiadis II, Levering WH, Roelen DL, Claas FH, Brand A. HLA-DRB1 associations in individuals with single and multiple clinically relevant red blood cell antibodies. *Transfusion*. 2014;54(8):1971–1980.
- Sippert EÁ, et al. Red blood cell alloimmunization in patients with sickle cell disease: correlation with HLA and cytokine gene polymorphisms. *Transfusion*. 2017;57(2):379–389.
- Calabro S, et al. Bridging channel dendritic cells induce immunity to transfused red blood cells. *J Exp Med*. 2016;213(6):887–896.
- Hendrickson JE, Chadwick TE, Roback JD, Hillyer CD, Zimring JC. Inflammation enhances consumption and presentation of transfused RBC antigens by dendritic cells. *Blood*. 2007;110(7):2736–2743.
- Arneja A, Salazar JE, Jiang W, Hendrickson JE, Zimring JC, Luckey CJ. Interleukin-6 receptor-alpha signaling drives anti-RBC alloantibody production and T-follicular helper cell differentiation in a murine model of red blood cell alloimmunization. *Haematologica*. 2016;101(11):e440–e444.
- Yazdanbakhsh K. Mechanisms of sickle cell alloimmunization. *Transfus Clin Biol*. 2015;22(3):178–181.

27. Bowman JM, Pollock JM, Manning FA, Harman CR, Menticoglou S. Maternal Kell blood group alloimmunization. *Obstet Gynecol.* 1992;79(2):239–244.
28. Eder AF. Update on HDFN: new information on long-standing controversies. *Immunohematology.* 2006;22(4):188–195.
29. Rimon E, et al. Management of Kell isoimmunization — evaluation of a Doppler-guided approach. *Ultrasound Obstet Gynecol.* 2006;28(6):814–820.
30. Smith NH, et al. Generation of transgenic mice with antithetical KEL1 and KEL2 human blood group antigens on red blood cells. *Transfusion.* 2012;52(12):2620–2630.
31. Stowell SR, et al. Anti-KEL sera prevents alloimmunization to transfused KEL RBCs in a murine model. *Haematologica.* 2015;100(10):e394–e397.
32. Girard-Pierce KR, et al. A novel role for C3 in antibody-induced red blood cell clearance and antigen modulation. *Blood.* 2013;122(10):1793–1801.
33. Stowell SR, et al. Transfusion of murine red blood cells expressing the human KEL glycoprotein induces clinically significant alloantibodies. *Transfusion.* 2014;54(1):179–189.
34. Polley MJ, Mollison PL. The role of complement in the detection of blood group antibodies: special reference to the antiglobulin test. *Transfusion.* 1961;1:9–22.
35. Harboe M, Mueller-Eberhard HJ, Fudenberg H, Polly MJ, Mollison PL. Identification of the components of complement participating in the antiglobulin reaction. *Immunology.* 1963;6:412–420.
36. Mollison PL. The role of complement in antibody-mediated red-cell destruction. *Br J Haematol.* 1970;18(3):249–255.
37. Mollison PL, Crome P, Hughes-Jones NC, Rochna E. Rate of removal from the circulation of red cells sensitized with different amounts of antibody. *Br J Haematol.* 1965;11:461–470.
38. Stowell SR, et al. Antigen modulation confers protection to red blood cells from antibody through Fcγ receptor ligation. *J Immunol.* 2013;191(10):5013–5025.
39. Liepkalns JS, Cadwell CM, Stowell SR, Hod EA, Spitalnik SL, Zimring JC. Resistance of a subset of red blood cells to clearance by antibodies in a mouse model of incompatible transfusion. *Transfusion.* 2013;53(6):1319–1327.
40. Madsen L, et al. Mice lacking all conventional MHC class II genes. *Proc Natl Acad Sci USA.* 1999;96(18):10338–10343.
41. Mombaerts P, et al. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature.* 1992;360(6401):225–231.
42. Pozdnyakova O, Guttormsen HK, Lalani FN, Carroll MC, Kasper DL. Impaired antibody response to group B streptococcal type III capsular polysaccharide in C3- and complement receptor 2-deficient mice. *J Immunol.* 2003;170(1):84–90.
43. Haas KM, et al. Complement receptors CD21/35 link innate and protective immunity during *Streptococcus pneumoniae* infection by regulating IgG3 antibody responses. *Immunity.* 2002;17(6):713–723.
44. Ahearn JM, et al. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity.* 1996;4(3):251–262.
45. Croix DA, et al. Antibody response to a T-dependent antigen requires B cell expression of complement receptors. *J Exp Med.* 1996;183(4):1857–1864.
46. Da Costa XJ, et al. Humoral response to herpes simplex virus is complement-dependent. *Proc Natl Acad Sci USA.* 1999;96(22):12708–12712.
47. Pepys MB. Role of complement in induction of the allergic response. *Nature New Biol.* 1972;237(74):157–159.
48. Carroll MC, Isenman DE. Regulation of humoral immunity by complement. *Immunity.* 2012;37(2):199–207.
49. Crotty S. A brief history of T cell help to B cells. *Nat Rev Immunol.* 2015;15(3):185–189.
50. Kawabe T, et al. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity.* 1994;1(3):167–178.
51. McAdam AJ, et al. ICOS is critical for CD40-mediated antibody class switching. *Nature.* 2001;409(6816):102–105.
52. Peng SL, Szabo SJ, Glimcher LH. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci USA.* 2002;99(8):5545–5550.
53. Lazarevic V, Glimcher LH, Lord GM. T-bet: a bridge between innate and adaptive immunity. *Nat Rev Immunol.* 2013;13(11):777–789.
54. King IL, Mohrs M. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med.* 2009;206(5):1001–1007.
55. Depoil D, et al. CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in response to membrane-bound ligand. *Nat Immunol.* 2008;9(1):63–72.
56. Ghannam A, et al. Human C3 deficiency associated with impairments in dendritic cell differentiation, memory B cells, and regulatory T cells. *J Immunol.* 2008;181(7):5158–5166.
57. Mattila PK, et al. The actin and tetraspanin networks organize receptor nanoclusters to regulate B cell receptor-mediated signaling. *Immunity.* 2013;38(3):461–474.
58. Rickert RC, Rajewsky K, Roes J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature.* 1995;376(6538):352–355.
59. Rother RP, Rollins SA, Mojcik CF, Brodsky RA, Bell L. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat Biotechnol.* 2007;25(11):1256–1264.
60. Strainic MG, et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity.* 2008;28(3):425–435.
61. Sheen JH, et al. TLR-induced murine dendritic cell (DC) activation requires dc-intrinsic complement. *J Immunol.* 2017;199(1):278–291.
62. Fang Y, Xu C, Fu YX, Holers VM, Molina H. Expression of complement receptors 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. *J Immunol.* 1998;160(11):5273–5279.
63. Heesters BA, et al. Endocytosis and recycling of immune complexes by follicular dendritic cells enhances B cell antigen binding and activation. *Immunity.* 2013;38(6):1164–1175.
64. Fischer MB, et al. Regulation of the B cell response to T-dependent antigens by classical pathway complement. *J Immunol.*

- 1996;157(2):549–556.
65. Molina H, et al. Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. *Proc Natl Acad Sci USA*. 1996;93(8):3357–3361.
66. Baiu DC, et al. Modulation of the humoral immune response by antibody-mediated antigen targeting to complement receptors and Fc receptors. *J Immunol*. 1999;162(6):3125–3130.
67. Fischer MB, et al. Dependence of germinal center B cells on expression of CD21/CD35 for survival. *Science*. 1998;280(5363):582–585.
68. Ansart-Pirenne H, Zeliszewski D, Lee K, Martin-Blanc S, Rouger P, Noizat-Pirenne F. Identification of immunodominant alloreactive T-cell epitopes on the Jka red blood cell protein inducing either Th1 or Th2 cytokine expression. *Blood*. 2004;104(10):3409–3410.
69. Stephen J, Cairns LS, Pickford WJ, Vickers MA, Urbaniak SJ, Barker RN. Identification, immunomodulatory activity, and immunogenicity of the major helper T-cell epitope on the K blood group antigen. *Blood*. 2012;119(23):5563–5574.
70. Fehr T, Skrastina D, Pumpens P, Zinkernagel RM. T cell-independent type I antibody response against B cell epitopes expressed repetitively on recombinant virus particles. *Proc Natl Acad Sci USA*. 1998;95(16):9477–9481.
71. Vos Q, Lees A, Wu ZQ, Snapper CM, Mond JJ. B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. *Immunol Rev*. 2000;176:154–170.
72. Mongini PK, Stein KE, Paul WE. T cell regulation of IgG subclass antibody production in response to T-independent antigens. *J Exp Med*. 1981;153(1):1–12.
73. Sato S, Miller AS, Howard MC, Tedder TF. Regulation of B lymphocyte development and activation by the CD19/CD21/CD81/Leu 13 complex requires the cytoplasmic domain of CD19. *J Immunol*. 1997;159(7):3278–3287.
74. Fearon DT, Carter RH. The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acquired immunity. *Annu Rev Immunol*. 1995;13:127–149.
75. Carroll M. Role of complement receptors CD21/CD35 in B lymphocyte activation and survival. *Curr Top Microbiol Immunol*. 1999;246:63–68.
76. Brantley SG, Ramsey G. Red cell alloimmunization in multitransfused HLA-typed patients. *Transfusion*. 1988;28(5):463–466.
77. Finck BK, Linsley PS, Wofsy D. Treatment of murine lupus with CTLA4Ig. *Science*. 1994;265(5176):1225–1227.
78. Kremer JM, et al. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N Engl J Med*. 2003;349(20):1907–1915.
79. Trouw LA, et al. Anti-cyclic citrullinated peptide antibodies from rheumatoid arthritis patients activate complement via both the classical and alternative pathways. *Arthritis Rheum*. 2009;60(7):1923–1931.
80. Basta M, Dalakas MC. High-dose intravenous immunoglobulin exerts its beneficial effect in patients with dermatomyositis by blocking endomysial deposition of activated complement fragments. *J Clin Invest*. 1994;94(5):1729–1735.
81. Birmingham DJ, et al. The complex nature of serum C3 and C4 as biomarkers of lupus renal flare. *Lupus*. 2010;19(11):1272–1280.
82. Leandro MJ, Edwards JC, Cambridge G, Ehrenstein MR, Isenberg DA. An open study of B lymphocyte depletion in systemic lupus erythematosus. *Arthritis Rheum*. 2002;46(10):2673–2677.
83. Kirou KA, Lee C, George S, Louca K, Peterson MG, Crow MK. Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. *Arthritis Rheum*. 2005;52(5):1491–1503.
84. Chen Z, Koralov SB, Gendelman M, Carroll MC, Kelsoe G. Humoral immune responses in Cr2^{-/-} mice: enhanced affinity maturation but impaired antibody persistence. *J Immunol*. 2000;164(9):4522–4532.
85. Phan TG, Grigorova I, Okada T, Cyster JG. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat Immunol*. 2007;8(9):992–1000.
86. Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science*. 1996;271(5247):348–350.
87. Patel SR, Smith NH, Kapp L, Zimring JC. Mechanisms of alloimmunization and subsequent bone marrow transplantation rejection induced by platelet transfusion in a murine model. *Am J Transplant*. 2012;12(5):1102–1112.
88. Wieland A, et al. Antibody effector functions mediated by Fcγ-receptors are compromised during persistent viral infection. *Immunity*. 2015;42(2):367–378.
89. Arthur CM, et al. CD8⁺ T cells mediate antibody-independent platelet clearance in mice. *Blood*. 2016;127(14):1823–1827.
90. Patel SR, Cadwell CM, Medford A, Zimring JC. Transfusion of minor histocompatibility antigen-mismatched platelets induces rejection of bone marrow transplants in mice. *J Clin Invest*. 2009;119(9):2787–2794.