

Anti-KEL sera prevents alloimmunization to transfused KEL RBCs in a murine model

Alloantibodies against red blood cell (RBC) antigens, which may be generated following exposure to foreign antigens on transfused RBCs or on fetal RBCs during pregnancy, can be clinically significant from the standpoint of morbidity and mortality.¹ In the transfusion setting, RBC alloantibodies can lead to premature clearance of transfused RBCs, resulting in hemolytic transfusion reactions and even death in severe cases. In the pregnancy setting, these antibodies can cause hemolytic disease of the fetus and newborn (HDFN). With the exception of RhIg, which is primarily utilized to prevent Rh(D) alloimmunization in pregnancy, no antigen specific targeted therapies exist to prevent RBC alloimmunization.

We hypothesized that a polyclonal antibody against another RBC specific antigen (KEL glycoprotein) may prevent KEL alloimmunization. The efficacy of RhIg was initially determined through administration to individuals transfused with Rh(D) positive RBCs,² and thus we opted to take the same approach using a model in which the human KEL glycoprotein antigen is present on murine RBCs.

Polyclonal anti-KEL glycoprotein alloantibodies were generated by transfusing wild-type C57BL/6 recipient

mice which lack the human KEL glycoprotein antigen with RBCs from transgenic KEL murine donors³ (Figure 1A and *Online Supplementary Appendix*). Serum was collected from recipients one month after the last of three biweekly transfusions, and the pooled sera composition was evaluated through a series of flow cytometric cross-matches with murine and human RBCs. The anti-KEL sera contained polyclonal anti-KEL glycoprotein IgG with reactivity against transgenic murine KEL RBCs but not wild-type C57BL/6 RBCs (Figure 1B). All IgG subtypes were represented, with essentially no detectable IgM (Figure 1C). Furthermore, the anti-KEL sera reacted against human reagent RBCs expressing the KEL2, Js^b, and Kp^b antigens or the KEL1, Js^b, and Kp^b antigens (Figure 1D, black histograms), with no reactivity after the KEL glycoprotein structure on the RBCs was disrupted via treatment with dithiothreitol (Figure 1D, gray histograms).

Following *in vitro* and *in vivo* dose optimization and clearance kinetic studies, anti-KEL sera or IgG enriched anti-KEL sera (*Online Supplementary Appendix*) were passively administered to C57BL/6 mice who were then transfused with the equivalent of one unit of KEL RBCs (Figure 2A). Whereas all recipients transfused with KEL RBCs in the absence of passively administered anti-KEL sera had detectable anti-KEL alloantibody responses, passive administration of anti-KEL sera or IgG enriched anti-KEL sera prior to RBC exposure completely prevented

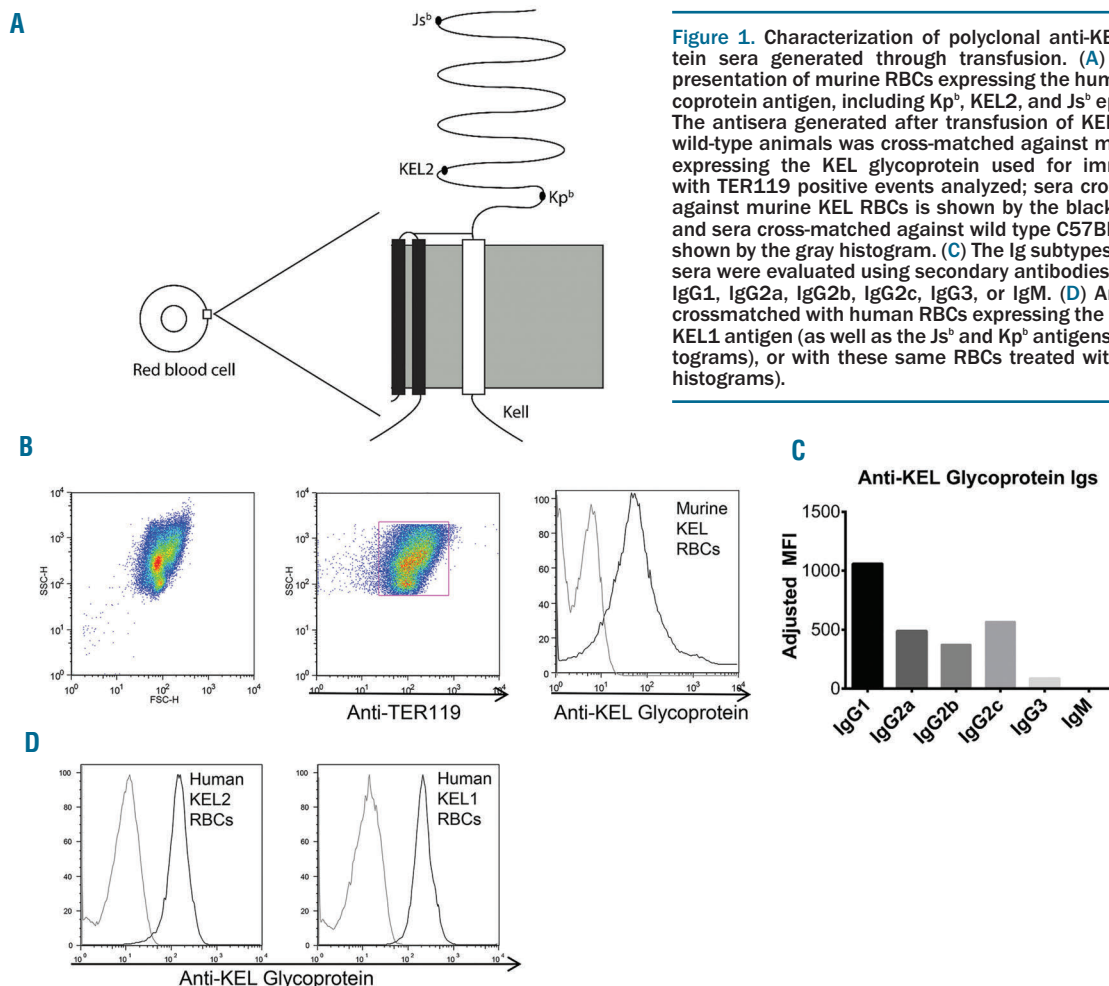


Figure 1. Characterization of polyclonal anti-KEL glycoprotein sera generated through transfusion. (A) Schematic presentation of murine RBCs expressing the human KEL glycoprotein antigen, including Kp^b, KEL2, and Js^b epitopes. (B) The antisera generated after transfusion of KEL RBCs into wild-type animals was cross-matched against murine RBCs expressing the KEL glycoprotein used for immunization, with TER119 positive events analyzed; sera cross-matched against murine KEL RBCs is shown by the black histogram and sera cross-matched against wild type C57BL/6 RBCs is shown by the gray histogram. (C) The Ig subtypes of the antisera were evaluated using secondary antibodies specific for IgG1, IgG2a, IgG2b, IgG2c, IgG3, or IgM. (D) Antisera was cross-matched with human RBCs expressing the KEL2 or the KEL1 antigen (as well as the Js^b and Kp^b antigens) (black histograms), or with these same RBCs treated with DTT (gray histograms).

C57BL/6 recipients from making detectable anti-KEL glycoprotein IgG at 16 weeks post-transfusion in 4 out of 4 experiments (Figure 2B and C). In contrast, passive transfer of anti-KEL sera to animals transfused with RBCs expressing a 3rd party antigen (HOD⁺) had no effect on recipient anti-HOD responses (*Online Supplementary Appendix*). Furthermore, passive transfer of anti-HEL sera had no effect on recipient anti-KEL responses to transfused KEL RBCs (Figure 2D). To investigate whether the infusion of anti-KEL sera impacted recipient anti-KEL immune responses long term, a subset of animals were re-transfused with KEL RBCs 16 weeks after initial anti-KEL sera treatment and KEL RBC transfusion. In contrast to the prevention of active alloimmunization noted when KEL RBCs were transfused in close proximity to anti-KEL sera infusion, all animals transfused 16 weeks after anti-KEL sera was infused generated levels of anti-KEL IgM and IgG similar to control animals (*Online Supplementary Appendix*).

To begin to investigate the mechanism(s) by which anti-KEL sera prevents alloimmunization, post-transfusion clearance studies were completed. Initial studies took a phenotypic approach (e.g. utilized anti-KEL

reagents to detect KEL RBCs) to investigate the persistence of transfused KEL RBCs; similar approaches are utilized by clinical transfusion services to determine the persistence of transfused RBCs in humans being evaluated for potential transfusion reactions. Taking this phenotypic approach, transfused KEL RBCs were detected for weeks post-transfusion in control animals, yet few transfused RBCs were detected beyond 24 h post transfusion in recipients treated with anti-KEL sera (*Online Supplementary Appendix*).

To track the transfused RBCs in a way that was not dependent on their KEL glycoprotein antigen expression, lipophilic dyes that covalently intercalate into the RBC membrane were utilized. KEL RBCs were labeled with DiO or with covalently modified DiI; a control tracker population of antigen negative C57BL/6 RBCs was labeled with the opposite dye in each study such that a ratio of KEL to control transfused RBCs could be analyzed in each post-transfusion sample. Parallel studies were completed with F1 blood donors expressing uGFP and KEL on their RBCs. Both of these approaches revealed that approximately 40%-50% of transfused KEL RBCs were cleared within 24 h in experimental mice

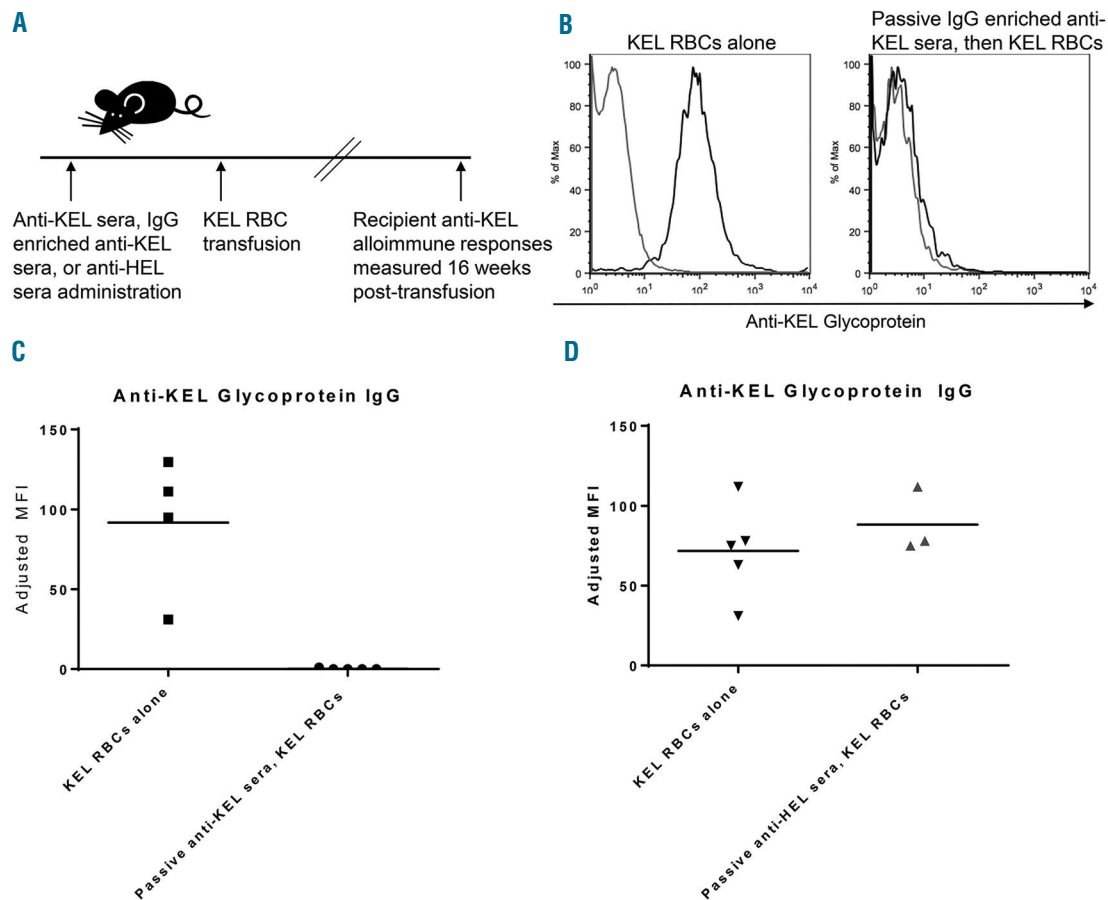


Figure 2. Anti-KEL sera prevent active alloimmunization to transfused KEL RBCs. (A) Schematic presentation of experimental design. (B) Representative anti-KEL glycoprotein IgG responses 28 days post-transfusion in the sera of mice transfused with KEL RBCs alone (left) or in those pre-treated with IgG enriched anti-KEL sera prior to transfusion (right); gray is signal of sera cross-matched with C57BL/6 RBCs and solid black is sera cross-matched with KEL RBCs. (C) Representative anti-KEL glycoprotein responses 28 days post-transfusion, with mean fluorescence intensity (MFI) being the difference between sera cross-matched with KEL or C57BL/6 RBCs. (D) Data from a representative experiment, in which recipients were transfused with KEL RBCs alone or pre-treated prior to transfusion with 3rd party sera (anti-HEL). Data shown are representative of 2-3 independent experiments, with 3-5 mice/group/experiment ($P < 0.05$ between groups in B and C; $P > 0.05$ in D).

treated with anti-KEL sera, with a plateau in preferential clearance of KEL RBCs within 24 h post-transfusion (Figure 3A-C and *Online Supplementary Appendix*). This clearance plateau was not due to a limiting amount of passively infused anti-KEL sera as no additional preferential clearance of KEL RBCs was observed when an excess of antisera was infused. Furthermore, the persistence of 50%-60% of KEL RBCs that remained in circulation beyond 24 h post transfusion was presumably not due solely to the recipient's reticuloendothelial system being saturated, as RBC dose titration experiments showed similar clearance patterns when 10-fold fewer RBCs were transfused (*Online Supplementary Appendix*).

These data suggest that in the presence of passively administered anti-KEL sera, the KEL antigen on transfused RBCs is either removed entirely or is modulated to the point of being undetectable. To further investigate these possibilities, more detailed analyses were completed on green fluorescent KEL RBCs recovered from the recipients that had been passively infused with anti-KEL sera; these results were compared to control animals transfused with KEL RBCs in the absence of anti-KEL

sera. Using polyclonal anti-sera as a detection reagent, the KEL antigen was largely undetectable on recovered green fluorescent KEL RBCs beyond 24 h post transfusion in animals passively infused with anti-KEL sera (Figure 3D). Direct antiglobulin tests (DATs) of recovered green fluorescent KEL RBCs were transiently positive post transfusion, but then negative beyond 24 h post transfusion in these animals (Figure 3E). Similarly, total complement was transiently detected on recovered green fluorescent KEL RBCs immediately post transfusion in animals passively infused with anti-KEL sera but not in control recipients (Figure 3F).

The importance of antigen modulation on the immunoprophylaxis effect of anti-KEL sera is not yet known, with ongoing experiments investigating this question. However, antigen modulation appears to be complete and non-reversible, with the KEL antigen remaining undetectable by flow cytometric phenotyping using polyclonal or monoclonal anti-Jsb or anti-Kpb reagents (data with monoclonal reagents not shown) for the remainder of the circulatory half-life of the transfused RBCs. In addition, intracellular RBC staining fails to

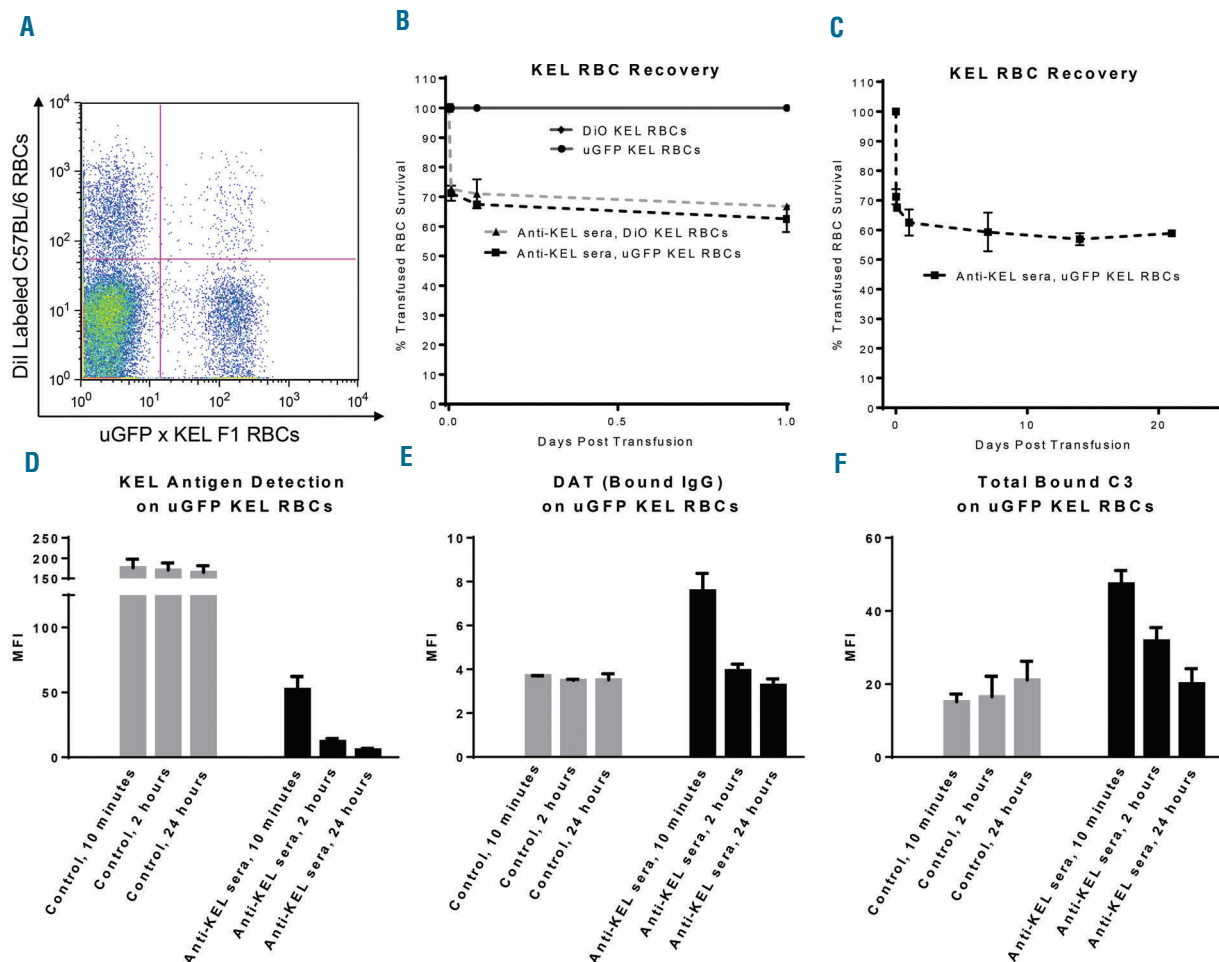


Figure 3. Anti-KEL sera results in RBC clearance and antigen modulation. (A) Representative flow plot from a transfusion recipient of naturally fluorescent (uGFP) KEL RBCs and control Dil labeled C57BL/6 RBCs. (B) Short-term KEL RBC post-transfusion recovery over time in recipients transfused with DIO KEL RBCs (gray lines) or uGFP KEL RBCs (black lines), in the presence or absence of anti-KEL sera. (C) Longer-term KEL RBC post-transfusion recovery in recipients transfused with uGFP KEL RBCs in the presence of anti-KEL sera. (D) KEL antigen detection, (E) Direct antiglobulin testing, and (F) Total bound C3 by flow cytometry over time from 10 min to 24 h post-transfusion, on uGFP KEL RBCs in recipients treated or not treated with anti-KEL sera. Data shown are representative of 3 experiments, with 3-5 mice/group/experiment. Error bars represent standard deviation.

reveal detectable KEL antigen in animals treated with anti-KEL sera prior to KEL RBC exposure (*data not shown*). Instability of the KEL glycoprotein antigen does not appear to be a contributing factor, with transgenic KEL RBCs transfused into agammaglobulinemic animals demonstrating stable KEL glycoprotein antigen expression long term (*Online Supplementary Appendix*). Antigen modulation is not unique to RBCs or murine models, with modulation of human WBC antigens being described⁵ and with modulation previously described in human RBC antigen systems⁶⁻⁹ and murine RBC antigen models.¹⁰⁻¹² However, the mechanism(s) of modulation appears to vary by antigen.

The phenomenon of antibody mediated immune suppression has been appreciated for many years, and has been studied primarily in non-RBC systems.¹³ However, the significance of complete cell clearance on antibody-mediated immune suppression continues to be a subject of debate, with prior sheep RBC studies providing little information in this regard due to the rapid base-line clearance of xenogenic RBC even in the absence of antibody. Historic studies of Rh(D) positive RBCs, transfused into male volunteers treated with RhIg, have led to the conclusion that complete RBC clearance is necessary for anti-D immunoprophylaxis.¹⁴ As a consequence, much attention has focused on preferentially developing monoclonal anti-D therapies that lead to rapid and complete clearance of chromium labeled Rh(D) positive RBCs.¹⁵ Taking our data into consideration, it is plausible that complete "antigenic" clearance is more critical than complete RBC clearance in the KEL system and potentially in other cognate antigen/antibody models, though structural differences between Rh(D) and the KEL glycoprotein must be taken into consideration.

In summary, we have demonstrated that passively administered polyclonal anti-KEL sera prevents alloimmunization to transfused murine RBCs expressing the entire human KEL glycoprotein. This is an antigen/antibody specific phenomenon, and the prophylactic effect does not persist after subsequent KEL RBC exposure. Our model provides a platform for future in depth mechanistic studies of antibody-mediated immune suppression, as well as for future studies investigating the efficacy of anti-KEL sera in the prevention of pregnancy-associated KEL alloimmunization. Knowledge gained from the KEL system, including that of anti-KEL sera leading to antigen modulation in the absence of complete RBC clearance, may inform other systems in which antibody prevention is desirable.

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