

## Transfusion of murine red blood cells expressing the human KEL glycoprotein induces clinically significant alloantibodies

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**BACKGROUND:** Red blood cell (RBC) alloantibodies to nonself antigens may develop after transfusion or pregnancy, leading to morbidity and mortality in the form of hemolytic transfusion reactions or hemolytic disease of the newborn. A better understanding of the mechanisms of RBC alloantibody induction, or strategies to mitigate the consequences of such antibodies, may ultimately improve transfusion safety. However, such studies are inherently difficult in humans.

**STUDY DESIGN AND METHODS:** We recently generated transgenic mice with RBC-specific expression of the human KEL glycoprotein, specifically the KEL2 or KEL1 antigens. Herein, we investigate recipient alloimmune responses to transfused RBCs in this system.

**RESULTS:** Transfusion of RBCs from KEL2 donors into wild-type recipients (lacking the human KEL protein but expressing the murine KEL ortholog) resulted in dose-dependent anti-KEL glycoprotein immunoglobulin (Ig)M and IgG antibody responses, enhanced by recipient inflammation with poly(I:C). Boostable responses were evident upon repeat transfusion, with morbid-appearing alloimmunized recipients experiencing rapid clearance of transfused KEL2 but not control RBCs. Although KEL1 RBCs were also immunogenic after transfusion into wild-type recipients, transfusion of KEL1 RBCs into KEL2 recipients or vice versa failed to lead to detectable anti-KEL1 or anti-KEL2 responses.

**CONCLUSIONS:** This murine model, with reproducible and clinically significant KEL glycoprotein alloantibody responses, provides a platform for future mechanistic studies of RBC alloantibody induction and consequences. Long-term translational goals of these studies include improving transfusion safety for at-risk patients.

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

Outside of transfusion avoidance or limited phenotypic or genotypic matching, very few therapeutic strategies exist to prevent or to minimize the dangers of RBC alloimmunization. To date, much has been learned about

**ABBREVIATIONS:** DiI = 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; DiO = 3,3'-dihexadecyloxycarbocyanine perchlorate; hGPA = human glyophorin; i.p. = intraperitoneally; mHEL = membrane-bound hen egg lysozyme.

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the functional characteristics and immunogenicity of individual human RBC antigens.<sup>3,4</sup> However, the numbers of variables involved in each transfusion make studying factors influencing the formation of alloantibodies difficult. Such variables may include donor and recipient health status, donor and recipient genetics and antigenic differences, and donor unit preparation and/or storage. Furthermore, ethical and practical considerations limit in-depth studies of the mechanisms of RBC alloantibody induction in humans. Finally, human studies involving RBC clearance patterns have historically been limited by the method (radioactivity) of RBC labeling, which allows a gross estimate of RBC clearance but limits in-depth analyses of individual transfused RBCs.

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

Each animal model has its own set of strengths and weaknesses; however, none recapitulates all aspects of a clinically significant human RBC antigen. The mHEL and Duffy<sup>b</sup> models have ubiquitous antigen expression, something not found with regards to many human RBC antigens; stringent removal of contaminating white blood cells and platelets is necessary for evaluation of RBC antigen responses. Anti-HEL generated in response to mHEL or HOD transfusions has been shown to result in selective removal of the HEL antigen instead of RBC clearance;<sup>19</sup> ongoing studies are further evaluating this phenomenon. The hGPA model results in anti-hGPA<sup>20</sup> when donors and recipients are MHC matched and when recipients are treated with poly(I:C), although anti-hGPA (e.g., anti-M family) in humans is rarely clinically significant. Interestingly, hGPA transfusion in the absence of inflammation leads to nonresponsiveness and potentially antigen-specific tolerance.<sup>15</sup> Passively infused anti-hGPA has been shown to lead to clearance of antigen-positive RBCs,<sup>21-24</sup> with mechanistic studies ongoing. Thus, much has been learned and much remains to be learned from these models; however, none to date results in a reproduc-

ible, robust, and boostable alloantibody response in the absence of an adjuvant, leading to RBC clearance with clinically significant sequelae.

We sought to develop a new model of RBC alloimmunization with the above characteristics, ideally utilizing an existing human antigen. The “Kell factor” was initially described half a century ago, after the direct antiglobulin test (DAT) was created and performed in instances after hydropic fetal complications.<sup>25</sup> Subsequently, anti-Kell family antibodies were shown to result in fatal hemolytic transfusion reactions.<sup>26</sup> Since that time, it has been shown that the Kell factor is actually a family of antigens, with Kell being a glycoprotein with endopeptidase activity.<sup>4</sup> Multiple epitopes on the Kell protein have been defined as clinically significant antigens, including Js<sup>a/b</sup> and Kp<sup>a/b</sup>.<sup>27</sup> The most well known, however, are KEL1 (also known simply as Kell) and KEL2 (also known as Cellano). Approximately 10% of transfusions are mismatched for KEL1/KEL2, with anti-KEL1 as well as anti-KEL2 leading to hemolysis of incompatible transfused RBCs or of incompatible fetal RBCs. In fact, KEL alloantibodies are a leading cause of transfusion- and pregnancy-associated morbidity and mortality today.<sup>28-33</sup>

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

## MATERIALS AND METHODS

### Mice

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD); KEL2 and KEL1 transgenic mice expressing the human KEL glycoprotein (previously published as “KEL2B” and “KEL1A”) were generated in our laboratory<sup>34</sup> and bred at Emory University (Atlanta, GA). All animals were housed in the Emory University Department of Animal Resources facilities, and all procedures

and protocols were approved by the Emory University Institutional Animal Care and Use Committee.

### Murine blood collection, fluorescent labeling, and transfusion

RBCs were collected into ACD and washed three times with phosphate-buffered saline (PBS) to remove residual citrate. Anti-KEL2 (Clone LKL1, Alba, Edinburgh, UK) or anti-KEL1 (Mima 23, provided by G. Halverson of the New York Blood Center, New York, NY) were utilized to confirm the presence of the KEL2 or KEL1 antigens on transfused RBCs. KEL2 RBCs were transfused in titrated amounts (0.5, 5, and 50  $\mu$ L of RBCs, diluted in PBS to 300  $\mu$ L total volume) via lateral tail vein; in some experiments, recipient animals were transfused every 2 weeks and in others, recipients were treated with 100  $\mu$ g of poly(I:C) (Amersham, Piscataway, NJ) given intraperitoneally (i.p.) 2 hours before transfusion. In a subset of experiments, RBCs were labeled with chloromethylbenzamido 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) or 3,3'-dihexadecyloxycarbocyanine perchlorate (DiO) according to the manufacturer's instructions as previously described (Molecular Probes, Eugene OR).<sup>24,36,37</sup> After labeling, experimental and control RBCs were mixed at a 1:1 ratio and recipient animals were transfused via lateral tail vein with 50 to 75  $\mu$ L of each blood type, diluted in PBS to 300  $\mu$ L total volume.

### Human RBC assays

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

### Flow cytometry

Serum was analyzed for the presence of anti-KEL immunoglobulin (Ig)M and IgG utilizing indirect immunofluorescence (flow cytometric crossmatch) with KEL2, KEL1, or control C57BL/6 RBCs; an adjusted mean fluorescence intensity (MFI) was calculated by subtracting the signal of serum cross-matched with antigen-negative targets from that of serum cross-matched with antigen-positive targets. Transfused RBCs were analyzed for presence of bound IgM and IgG using allophycocyanin-conjugated goat anti-mouse IgM and Alexa Fluor 488-conjugated goat anti-mouse IgG, respectively (Jackson ImmunoResearch, West Grove, PA), after gating on the lipophilically labeled RBCs. Posttransfusion KEL2 RBC recovery and survival were determined by flow cytometry and lipophilic dye fluorescence, utilizing a ratio of transfused KEL2 RBCs to

control C57BL/6 RBCs. All antibodies were used at 1:100 dilution and all samples were analyzed on a four-color flow cytometer (FACSCalibur, BD, Franklin Lakes, NJ).

### Cytokine analysis

Serum cytokines, including interleukin (IL)-6, keratinocyte-derived chemokine (the murine equivalent to IL-8 in humans), monocyte chemoattractant protein-1, macrophage inflammatory protein-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , and interferon (IFN)- $\gamma$ , were evaluated 90 to 120 minutes posttransfusion using the cytometric bead array mouse flex kit (BD Biosciences, San Diego, CA).

### Statistical analysis

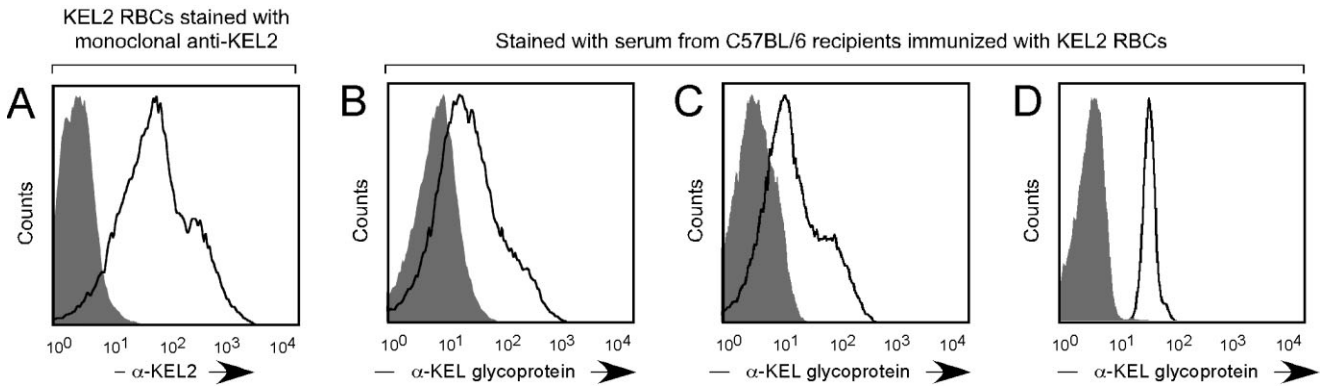
Statistical significance was determined by performing a t test for comparison of two groups or one-way analysis of variance with Tukey's posttest when three groups were compared using computer software (Prism, GraphPad, San Diego, CA). Error bars represent one standard deviation (SD) and differences were considered significant with p values of 0.05 or less.

## RESULTS

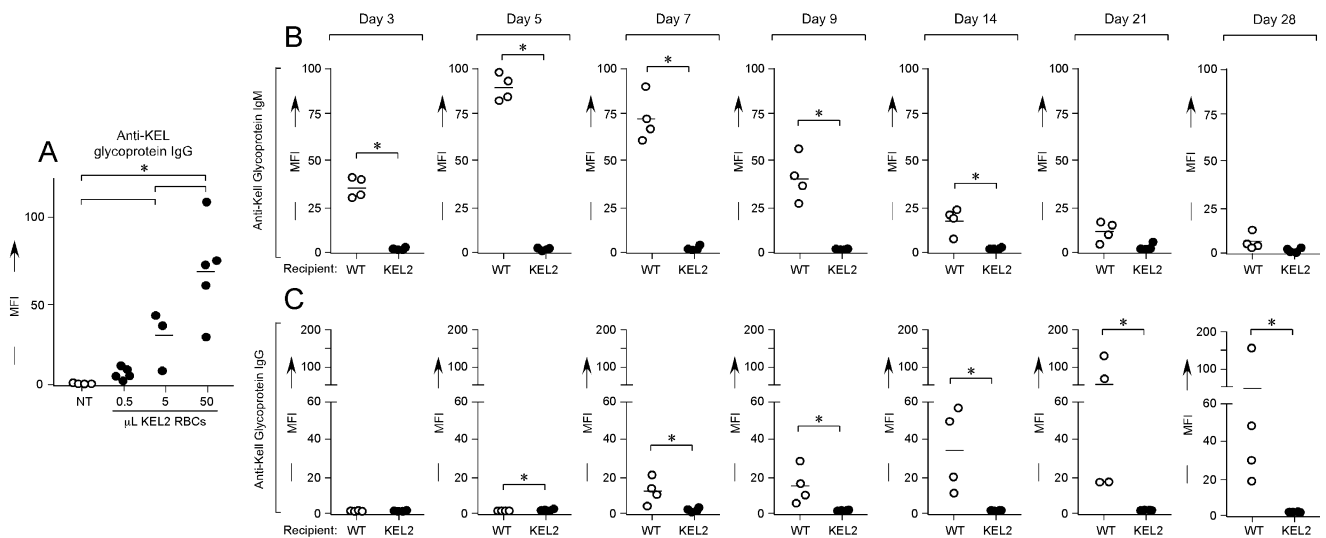
### Serologic responses of C57BL/6 recipients to transfused KEL2 RBCs

Blood was collected from KEL2 donors, and the presence of KEL2 expression on the pooled RBCs was determined before transfusion using monoclonal anti-KEL2 (Fig. 1A). The equivalent of 1 human "unit" of KEL2 RBCs (50-75  $\mu$ L) was then transfused into wild-type C57BL/6 recipients. Fourteen to 21 days posttransfusion, recipient serum was cross-matched with KEL2 or wild-type control C57BL/6 RBC targets, using anti-mouse IgG as a secondary reagent (Fig. 1B shows a representative cross-match after three transfusions). An adjusted MFI was calculated by subtracting the background signal of serum cross-matched with wild-type RBCs from that of serum cross-matched with KEL2 RBCs. The only known difference between wild-type C57BL/6 and KEL2 RBC targets is the KEL glycoprotein antigen,<sup>34</sup> thus eliminating MHC mismatch as a variable and leading one to hypothesize that the observed adjusted MFI signal is due to anti-KEL glycoprotein alloantibodies.

To further evaluate the specificity of the anti-KEL glycoprotein antibody response, serum from immunized mice was cross-matched with murine KEL2 RBCs or murine KEL2 RBCs treated with 0.2 mol/L DTT (Fig. 1C). DTT is known to reduce disulfide bonds to free sulfhydryl groups, with disulfide bonds being a requirement for KEL antigen integrity.<sup>38</sup> These serum cross-matches were repeated using human RBCs expressing the KEL2 antigen or human RBCs expressing the KEL2 antigen that had been treated with 0.2 mol/L DTT as targets (Fig. 1D). In



**Fig. 1.** Transgenic murine RBCs express the human KEL2 antigen, and C57BL/6 recipients of KEL2 RBC transfusion make alloantibodies with KEL specificity. (A) Murine RBCs from KEL2-transgenic donors were collected and stained with anti-KEL2 before transfusion. (■) Mouse C57BL/6 RBCs; (—) mouse KEL2 RBCs. (B) Serum from C57BL/6 recipients transfused with KEL2 RBCs was cross-matched with murine KEL2 RBCs (—) or wild-type C57BL/6 RBCs (■). (C) Serum from C57BL/6 recipients transfused with KEL2 RBCs was cross-matched with murine KEL2 RBCs (—) or with DTT-treated murine KEL2 RBCs (■). (D) Serum from C57BL/6 recipients transfused with KEL2 RBCs was cross-matched with human RBCs expressing KEL2 (—) or with DTT-treated human RBCs (■); representative results are shown.

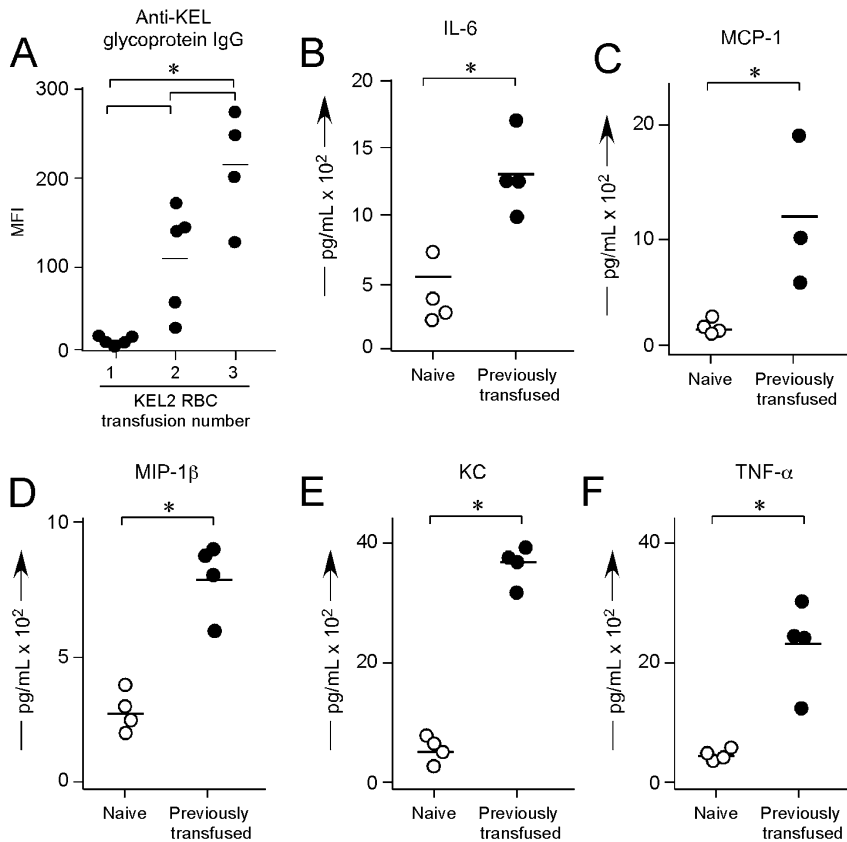


**Fig. 2.** C57BL/6 recipients lacking human KEL have a dose-dependent anti-KEL glycoprotein antibody response. (A) Control (KEL2) or wild-type (WT) C57BL/6 recipients were transfused with 0.5, 5, or 50  $\mu$ L of KEL2 RBCs, with serum anti-KEL glycoprotein IgG evaluated 2 weeks after transfusion. Serial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

both instances, the observed reactivity with intact KEL2 expressing murine or human target RBCs was essentially eliminated upon cross-match with DTT-treated target RBCs. These results lend further support to the KEL specificity of the alloantibody observed in the sera of C57BL/6 recipients after transfusion with transgenic murine KEL2 RBCs.

To evaluate dose responses to transfused KEL RBCs, 0.5, 5, or 50  $\mu$ L of KEL2 RBCs were transfused into C57BL/6 recipients, with serum evaluated 2 weeks later by flow

cross-match with transgenic murine KEL2 or C57BL/6 RBCs. In two of two experiments ( $n = 27$  animals total), a dose-titratable anti-KEL glycoprotein IgG response was observed (Fig. 2A shows adjusted MFI). For the remainder of the experiments described herein, 50  $\mu$ L of RBCs were thus utilized for transfusion. To more completely characterize the immune response of C57BL/6 recipients to transfused KEL2 RBCs, wild-type or KEL2 syngeneic recipients were transfused with 50  $\mu$ L of KEL2 RBCs, and anti-KEL IgM and IgG in the serum was measured by flow



**Fig. 3.** C57BL/6 recipients have “boostable” responses to repeat KEL2 exposure, with a proinflammatory serum cytokine storm. (A) C57BL/6 recipients were transfused every 2 weeks with KEL2 RBCs, with serum anti-KEL glycoprotein immunoglobulins evaluated on Day 14 after each transfusion. (B-F) Serum cytokine responses in alloimmunized animals, 90 to 120 minutes after a fourth KEL2 RBC transfusion. Results are representative of two to three independent experiments with at least three to five mice per group; \* $p < 0.05$ . KC = keratinocyte-derived chemokine; MCP-1 = monocyte chemoattractant protein-1; MIP-1 $\beta$  = macrophage inflammatory protein-1 $\beta$ .

cross-match from Days 3 through 28 posttransfusion. In a compilation of three independent experiments ( $n = 24$  animals total), anti-KEL IgM was observed in the serum of C57BL/6 but not syngeneic recipients by Day 3 posttransfusion, peaking by Day 5. Anti-KEL IgG was observed in the serum of C57BL/6 but not syngeneic recipients by Day 7 posttransfusion, peaking near Days 14 to 21 (Figs. 2B and 2C show a representative experiment).

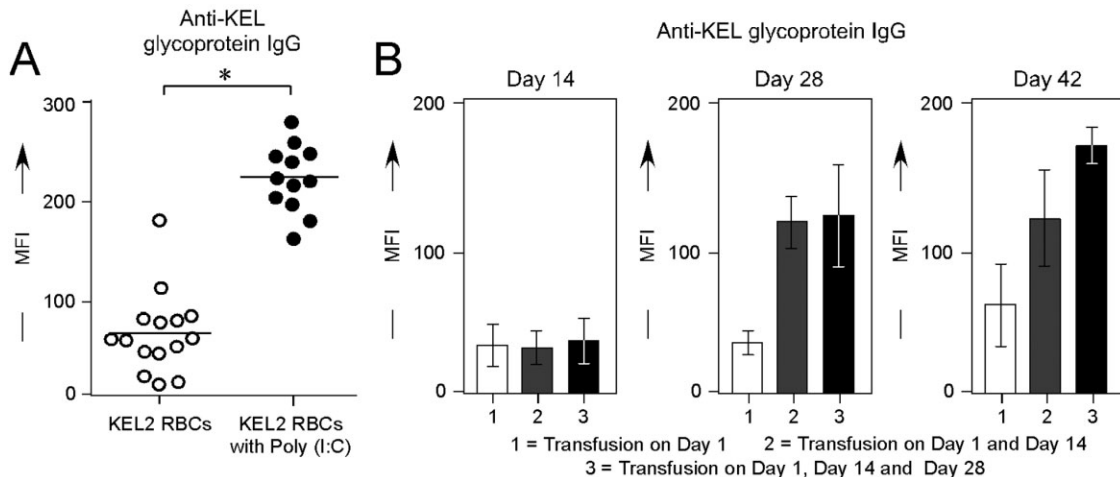
#### Assessment of repeat antigen exposure and cytokine responses

Given the reported evanescence patterns of RBC alloantibodies in humans,<sup>39,40</sup> antigen-positive RBCs may be unintentionally transfused into humans with alloantibodies that fall below the level of detection by traditional blood bank antibody screening methods. Anamnestic responses

may subsequently result, with a rapid increase in RBC alloantibody titer and premature clearance of transfused RBCs. In fact, some such transfusions may lead to such significant hemolysis of both transfused and “bystander” RBCs that renal failure, disseminated intravascular coagulation, and death result.<sup>41</sup> Past efforts by our laboratory to create murine models with antibody responses that are “boostable” in the absence of coexistent recipient inflammation have thus far not been successful, likely due to characteristics of the antigens previously studied. To examine potential boostable responses in our newly developed KEL system, KEL2 RBCs were transfused every 2 weeks for a total of three transfusions into C57BL/6 recipients, with total anti-KEL immunoglobulins measured 2 weeks after each transfusion. In three of three experiments ( $n = 15$  recipients total), significant increased responses were observed after each transfusion (Fig. 3A shows a representative experiment). This increased alloantibody response was not simply due to a continued increase in anti-KEL titer over time, as control animals receiving a single transfusion had peak alloantibody responses at approximately 14 to 21 days posttransfusion.

In humans, incompatible RBC transfusions are the second leading cause of transfusion-associated death.<sup>1</sup> To date, however, few animal models have been created in which repeat

transfusions not only lead to high-titer alloantibodies but also to adverse clinical outcomes. Two laboratories have reported an animal model in which anti-hGPA resulted in clearance of incompatible RBCs and a proinflammatory serum cytokine storm;<sup>21-24,36</sup> however, these antibodies were passively infused and not actively generated. In our current studies, C57BL/6 recipients were actively alloimmunized through repeat KEL2 transfusions. These alloimmunized recipients were then retransfused a fourth time with “incompatible” KEL2 RBCs and observed. Within 5 minutes of the incompatible transfusion, recipients appeared ill and hunched over. Cytokine bead arrays were performed on recipient serum 90 to 120 minutes posttransfusion, revealing significant elevations of IL-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1 $\beta$ , keratinocyte-derived chemokine, and TNF- $\alpha$  in alloimmunized animals receiving



**Fig. 4. Recipient inflammation with poly(I:C) enhances anti-KEL responses, with “boostable” responses. (A) Serum anti-KEL glycoprotein IgG responses in C57BL/6 animals transfused with KEL2 RBCs in the presence or absence of recipient poly(I:C) treatment; straight serum, 14 days after transfusion. (B) Serum anti-KEL glycoprotein IgG responses after one, two, or three KEL2 RBC transfusions in the presence of poly(I:C); sera diluted 1:10, tested every 14 days. Results are representative of two to three experiments with three to five mice per group; \* $p < 0.05$ .**

incompatible RBCs; no changes were observed in IFN- $\gamma$  (Figs. 3B-3F show representative data).

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

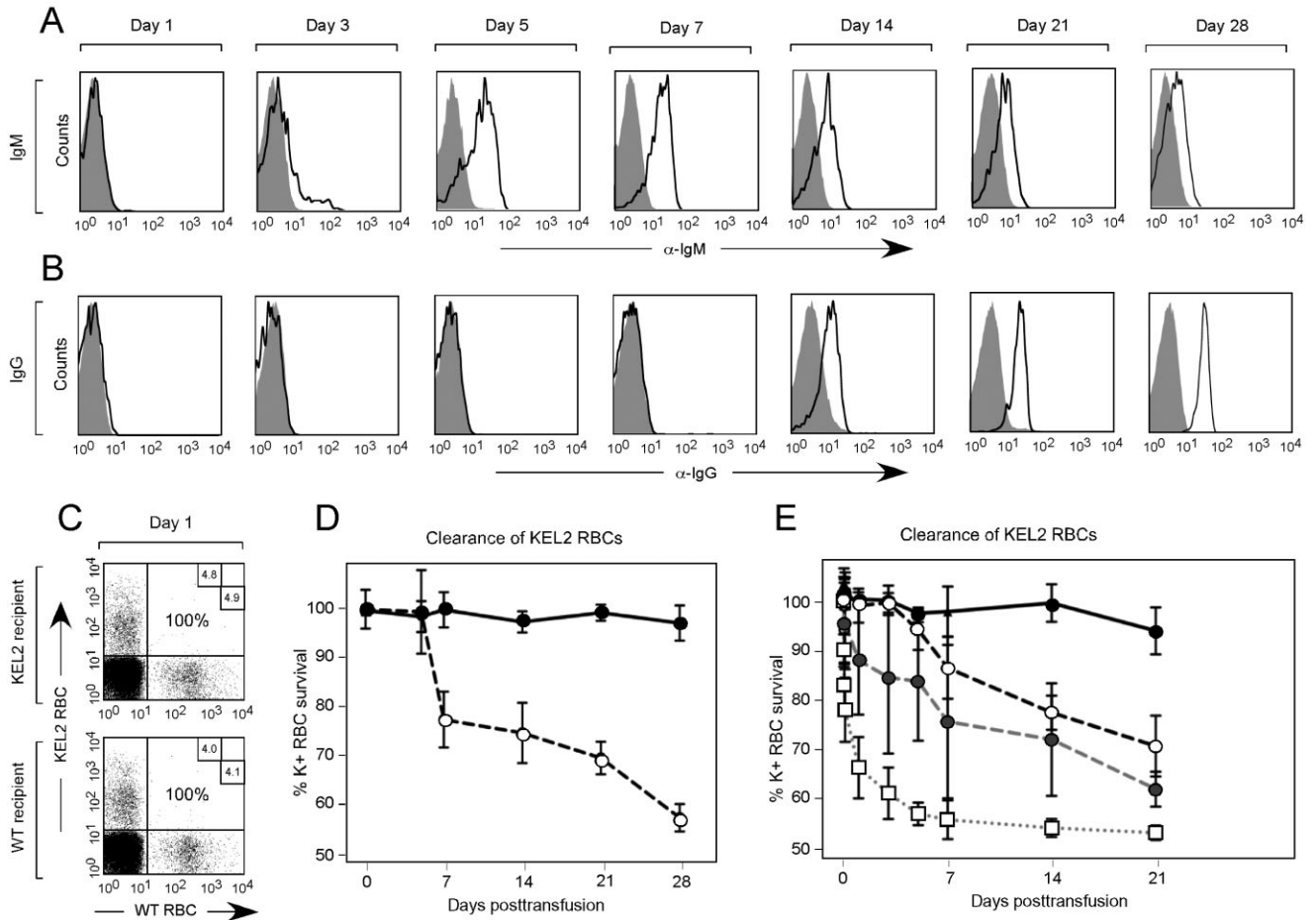
It is known that a “danger” signal of sorts leads to an augmented immune response in a number of settings, both clinical and experimental.<sup>42</sup> We have previously reported that recipient inflammation with the double-stranded RNA poly(I:C) enhances RBC alloimmunization in multiple different murine RBC alloimmunization models, including the mHEL, hGPA, and HOD models.<sup>15,43,44</sup> To evaluate the effect of recipient inflammation on alloimmunization in our KEL model, C57BL/6 recipient animals were treated with 100  $\mu$ g of poly(I:C) i.p.; control animals were treated with PBS i.p. Subsequently, all recipients were transfused with KEL2 RBCs and serum alloantibody responses were evaluated at multiple time points posttransfusion. In three of three experiments ( $n = 27$  animals total), poly(I:C)-treated animals had significantly higher serum anti-KEL IgG levels posttransfusion than control animals (Fig. 4A shows compilation data on Day 14 posttransfusion,  $p < 0.05$ ). To determine boostable responses in the presence of poly(I:C), three separate cohorts of C57BL/6 recipients were transfused one, two, or three times in the presence of poly(I:C). Serum samples were evaluated every 2 weeks in each cohort, out 42 days from the time of the initial transfusion. Similar to what was observed in the absence of poly(I:C), boostable responses were observed in the presence of poly(I:C) with peak titers noted approximately 2 weeks posttransfusion (Fig. 4B shows representative data). These responses were

not an artifact of increased anti-KEL responses over time, as control animals receiving a single transfusion did not have significant increases in anti-KEL beyond 14 to 21 days posttransfusion (Fig. 4B shows three distinct cohorts of animals receiving one, two, or three transfusions, with anti-KEL glycoprotein antibody responses measured over time in each cohort).

#### Anti-KEL RBC binding and clearance

The studies described to this point have focused on serologic evaluation of transfused recipients. Serum is the focus of clinical transfusion medicine evaluations, given difficulties in evaluating antibody bound to transfused RBCs. In fact, “mixed-field” reactions, in which a subset of recipient RBCs (e.g., those transfused) have bound antibody whereas another subset of recipient RBCs (e.g., the patient’s own RBCs) lack bound antibody, can be quite technically challenging to evaluate. Practically speaking, however, an evaluation of the RBC antibody bound *in vivo* may be a better predictor of the fate of the transfused RBCs than an *in vitro* test of serum alloantibodies.

To allow visualization of cells in the recipient after transfusion, KEL2 RBCs were labeled before transfusion with a lipophilic dye (DiI) that intercalates into the lipid RBC membrane; control C57BL/6 RBCs were labeled with a dye that fluoresces on a different channel (DiO). These dyes have been carefully dose titrated, such that the RBC circulatory half-life remains unaffected.<sup>34</sup> KEL2 and C57BL/6 RBCs were mixed and transfused. DATs for IgM and IgG were performed at multiple time points after transfusion on transfused DiI-positive KEL2 RBCs, comparing signal on transfused DiI-positive KEL2 RBCs to that



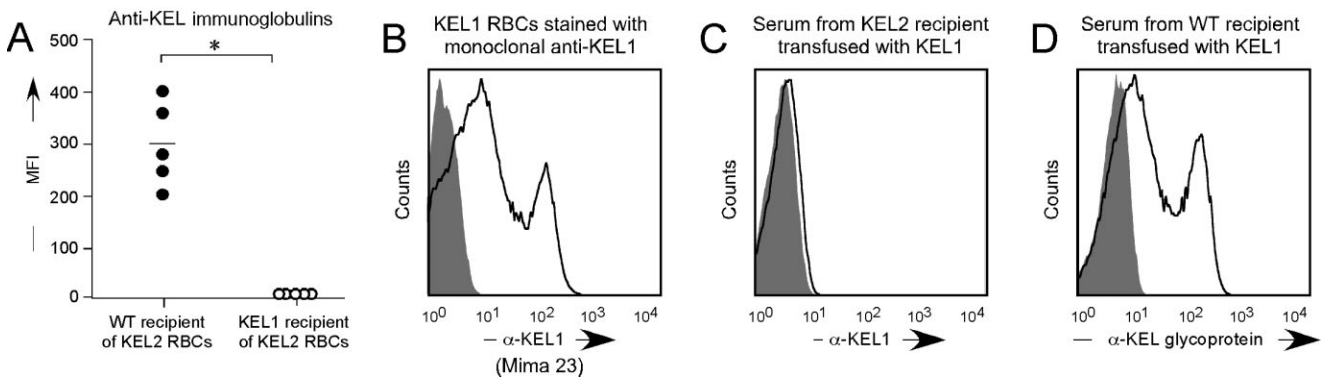
**Fig. 5.** Circulating anti-KEL glycoprotein immunoglobulins bind to transfused KEL2 RBCs and are associated with KEL2 RBC clearance. KEL2 and C57BL/6 RBCs were labeled with DiI and DiO, respectively, before transfusion. RBC-bound IgM (A) and IgG (B) were evaluated serially after transfusion on DiI-positive KEL RBCs; shaded histograms are control antigen-negative cells. Posttransfusion survival and recovery of KEL2 RBCs was determined by comparing a ratio of DiI KEL to DiO C57BL/6 RBCs (C). These studies were completed in KEL2 and wild-type (WT) C57BL/6 recipients (D; ●, KEL2 recipients; ○, WT recipients); similar studies were also completed after a second KEL2 transfusion in recipients initially transfused with or without poly(I:C) (E; ●, KEL2 recipients; ○, WT recipients; ■, WT retransfusion; □, WT PIC retransfusion). Error bars represent SD and results represent at least three independent experiments with three to five mice per group.

of control C57BL/6 RBCs (Figs. 5A and 5B). In three of three experiments (n = 24 animals total), anti-KEL glycoprotein IgM and IgG were detected on circulating KEL2 RBCs by approximately Days 3 and 7 posttransfusion, respectively. The timing of the initial detection of anti-KEL bound to circulating RBCs thus closely parallels that of anti-KEL detected in serum.

Transfused recipients were also serially evaluated after transfusion for clearance of KEL2 RBCs. A ratio of DiI-positive KEL2 RBCs to DiO-positive C57BL/6 RBCs was determined at multiple time points after transfusion (Fig. 5C) in syngeneic KEL2 recipients or wild-type naive C57BL/6 recipients. Although little to no detectable KEL-specific RBC clearance was observed in syngeneic KEL2 recipients to which KEL2 is a self-antigen, a slow relative decline in the ratio of transfused KEL2 to C57BL/6 RBCs

was appreciated in wild-type C57BL/6 recipients over a 28-day period (Fig. 5D depicts a representative experiment with three to five animals/group).

Given the morbid appearance and serum cytokine storm observed in alloimmunized animals transfused with incompatible KEL2 RBCs, we hypothesized that KEL-specific RBC clearance would rapidly occur in such a setting. To test this hypothesis, alloimmunized or naive C57BL/6 recipients were transfused with DiI-labeled KEL2 and DiO-labeled C57BL/6 RBCs, with posttransfusion KEL2 RBC recovery and survival tracked. As hypothesized, the incompatible KEL2 RBCs were cleared more rapidly after transfusion in alloimmunized than naive C57BL/6 recipients (Fig. 5E depicts a representative experiment with three to five animals/group). Furthermore, recipients who were initially treated with poly(I:C) and transfused,



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

with demonstrable higher-titer anti-KEL glycoprotein alloantibodies, had subtly higher rates of KEL-specific RBC clearance (Fig. 5E). This clearance was not an artifact of decreased stability of the KEL2 RBCs in general, however, as these RBCs had a normal circulatory half-life in syngeneic KEL2 recipients.

### Serum responses to single amino acid polymorphisms on murine RBCs

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

Additional studies were completed using KEL1 RBCs transfused into KEL2 recipients, which is the scenario most likely to be encountered in human clinical setting. We have previously shown stable KEL1 antigen expression, epitope integrity, and posttransfusion recovery or survival in these animals;<sup>34</sup> Fig. 6B shows KEL1 transgenic RBCs stained with a monoclonal anti-KEL1 reagent (Mima 23, provided by G. Halverson, New York Blood Center). KEL1 RBCs were transfused every 2 weeks  $\times 3$  into KEL2 recipients, in the presence of poly(I:C). As described in the KEL2 donor/KEL1 recipient setting, the KEL1 donor/KEL2 recipient setting also failed to generate detectable serum

anti-KEL1 responses (Fig. 6C). This lack of a response was not due to KEL1 RBCs being incapable of being immunogenic, as wild-type C57BL/6 recipients made detectable anti-KEL glycoprotein antibodies after KEL1 RBC transfusion (Fig. 6D).

## DISCUSSION

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

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



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

In addition to studies of immunity induction, this KEL model allows for studies of the fate of transfused RBCs. Human studies utilizing chromium labeling are somewhat limited to blunt evaluations of clearance curves. The strategy of labeling RBCs with a lipophilic dye, as previously described by our group, allows for in-depth flow cytometric evaluation of the transfused cells. A slow but reproducible pattern of KEL2-specific RBC clearance was noted after an initial transfusion, with more rapid clearance and a proinflammatory serum cytokine response observed in previously alloimmunized recipients with higher-titer alloantibodies. The degree of intravascular versus extravascular hemolysis cannot be evaluated from the current studies, as extensive blood or urine chemistry analyses were not completed. However, there was no appreciable bystander hemolysis of the recipient's own RBCs, as hematocrit values were noted to be similar in alloimmunized and nonalloimmunized recipients of KEL2 RBCs (data not shown). Clearance patterns correlated with detectable serum and RBC bound anti-KEL immunoglobulins, with no clearance of antigen-negative C57BL/6 RBCs noted. However, in no experiment were all transfused KEL2 RBCs cleared by Day 28. These findings suggest that KEL2 RBCs remaining in circulation may be acquiring a survival advantage of sorts. We have previously reported RBC populations resistant to hemolysis in the hGPA system.<sup>36</sup> In addition, KEL antigens are known to have the potential to undergo weakened antigenicity during incompatibility in human settings;<sup>45,46</sup> ongoing studies are investigating clearance as well as clearance resistance mechanisms in our model.

Limitations to the KEL into C57BL/6 model and experiments described must be considered. One such consideration is that this model resembles that of D in humans more so than that of KEL1/KEL2, in that the human KEL antigen is present on donor RBCs but lacking on recipient RBCs. This is, perhaps, more of a deviation from what is "human" than a true scientific limitation of the model; findings from this described model will likely have applicability to some but not all antigens. Other considerations include the potential confounders involved in utilizing lipophilic dyes to track transfused RBCs; despite careful titration and control studies, their impact on study

results cannot definitively be ruled out. Another consideration is that poly(I:C) is just one type of recipient inflammation, and other toll-like receptor agonists<sup>43</sup> or authentic infections may have differential effects on alloimmunization outcomes. A last consideration is that these studies were completed utilizing KEL2 and KEL1 animals, which have approximately 1200 and 800 copies of KEL glycoprotein per RBC.<sup>34</sup> Additional KEL transgenic animals have since been created with lower and higher RBC antigen copy numbers, with ongoing studies investigating the impact of copy number and dose responses on alloimmunogenicity and clinical consequences.

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

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#### CONFLICT OF INTEREST

The authors declare they have no conflicts of interest related to this work; the transgenic animals were generated in part by funding from Immucor to JCZ.

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