

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

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## **Supplemental Methods:**

### **Mice**

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Triple congenic B6.Cg-Gpi1<sup>a</sup>Th1<sup>a</sup>IgH<sup>a</sup>/J mice, as well as mice expressing green fluorescence protein behind a ubiquitin promoter (C57BL/6-Tg(UBC-GFP)30Scha/J,uGFP) were purchased from Jackson Laboratories (Bar Harbor, ME). *KEL2* transgenic mice expressing the human KEL glycoprotein including the *KEL2* polymorphism were generated and bred by our laboratory(1); these animals, which are heterozygous for the KEL antigen, were crossed with uGFP mice for some of the experiments. The *KEL2* mice used for these experiments have been previously described as “KEL2B,” and have a mean copy number of approximately 1000 *KEL2* molecules on the RBC cell surface; in this manuscript they are referred to as “KEL” mice for simplicity given that the antigen being studied includes the entire human KEL glycoprotein(2). HOD mice expressing a fusion protein containing the hen egg lysozyme, ovalbumin, and the human Duffy<sup>b</sup> antigens were also generated as previously described (3). All animals were housed in Emory or Yale University’s Animal Facilities.

### **Antibodies and immunization**

Antiserum against the KEL glycoprotein was generated by transfusing *KEL2* RBCs into triple congenic IgH<sup>a</sup> recipients pre-treated with an intraperitoneal injection of 100µg poly (I:C) (Amersham/GE Healthcare, NY) a total of 3 times, separated by two weeks between each injection. Pooled sera collected 2-4 weeks after the final transfusion was tested for KEL binding

ability by flow crossmatch with KEL2 or control C57BL/6 RBCs as targets, and using APC-conjugated goat anti-mouse IgG (BD Biosciences) or Biotin goat anti-mouse IgM or IgG subtypes (Bethyl Laboratories, Montgomery, TX), plus anti-SA conjugated PercP-Cy5.5 (Jackson ImmunoResearch Laboratories, West Grove, PA).

For some experiments, sera from immunized mice enriched in IgG was generated by purification over a Protein A column (Pierce Biotechnology, Thermo Fisher Scientific, Rockville, IL), according to manufacturer instructions. Briefly, total serum was mixed 1:1 with binding buffer (0.1M phosphate, 0.15M sodium chloride, pH 7.2), and added to Protein A. The column was washed with 10 volumes of binding buffer before elution buffer (0.1M glycine-HCL, pH 2.5-3) was added to elute bound IgG. Antibody was eluted directly into neutralization buffer (1M Tris, pH 8-9), and positive fractions were pooled and passed over a PD10 column equilibrated with PBS (pH 7.4). SDS-PAGE of total serum, flow through, wash fractions, and 2 µg of purified anti-KEL sera enriched in IgG was completed.

In passive immunization experiments, recipient mice were given 25-50 µl of anti-KEL sera or 25 µg of anti-KEL sera enriched in IgG intravenously 2 hours prior to transfusion. Antibodies against the HEL antigen (anti-HEL sera) were generated by immunizing recipients with soluble HEL emulsified with complete Freund's adjuvant. Monoclonal antibodies against the Jsb (Mima 8) and Kpb (Mima 9) epitopes of the KEL glycoprotein were purchased from the NY Blood Center, and polyclonal anti-KEL2 reagent was purchased from Ortho-Clinical Diagnostics, Inc. (Raritan, NJ).

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

Donor KEL2, HOD, or wild type C57BL/6 RBCs were collected into acid citrate dextrose (ACD) and washed three times to remove residual citrate. Prior to transfusion, RBCs were labeled with chloromethylbenzamido 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI) or 3,3'-dihexadecyloxycarbocyanine perchlorate (DiO) according to the manufacturer's instructions (Molecular Probes, Eugene OR) and as previously described(4). After labeling, cells were washed at least three times to remove any unbound dye. Experimental and control RBCs were mixed at a 1:1 ratio and recipient mice were transfused via lateral tail vein with 50  $\mu$ l of each type of blood (equivalent of one human unit). Survival of the transfused RBCs was determined by comparing the ratio of circulating KEL2 or HOD RBCs to control RBCs in recipients at select time points ranging from 10 minutes to 35 days post-transfusion.

### **Flow cytometry**

To evaluate the active immune response to mice after transfusion, serum was collected at multiple time points and crossmatched with antigen positive RBCs (KEL or HOD) or control C57BL/6 RBCs. Secondary antibodies included goat anti-mouse immunoglobulin conjugated to allophycocyanin (BD Biosciences, San Jose, CA) or F(ab')<sub>2</sub> fragment IgM mu chain specific (Jackson ImmunoResearch, West Grove, PA); the antigen specific response (adjusted MFI) was determined by subtracting the signal of serum with C57BL/6 RBCs from that of serum with antigen positive RBCs. Other flow cytometric crossmatches were completed using IgH<sup>a</sup>/IgH<sup>b</sup> specific secondary reagents including anti-mouse IgG1a(a), IgG2a(a), IgG1a(b) and IgG2a(b) (BD Biosciences, San Jose, CA). Additional antisera characterization was completed using murine RBCs as well as human screening RBCs (Biotestcell, Bio-Rad Medical Diagnostics,

Dreieich, Germany); 0.2 M dithiothrietol (Sigma-Aldrich, St. Louis, MO) was utilized to reduce disulfide bonds on select human screening RBCs prior to staining.

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

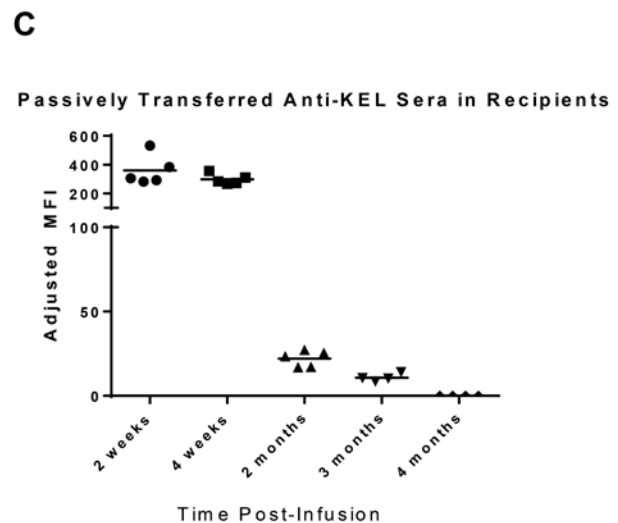
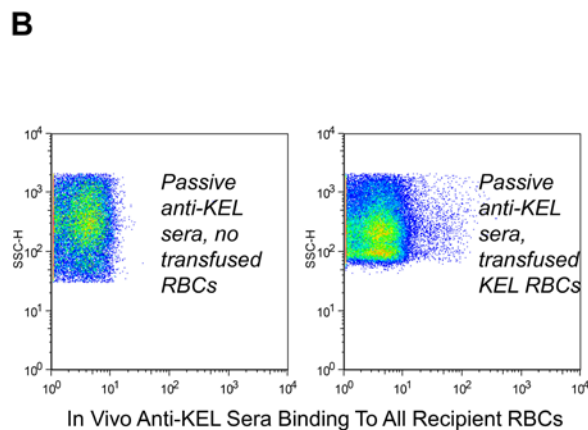
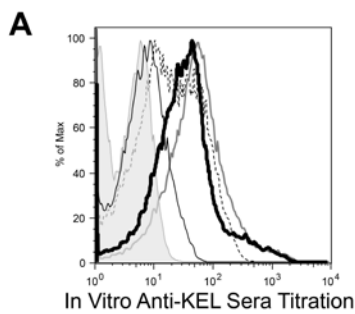
## **Statistics**

All statistical analysis was performed using Graph Pad Prism software (San Diego, CA). A Mann Whitney U test was used to determine significant differences between two groups. Error bars represent one standard deviation, and significance was determined by a p-value of  $\leq 0.05$ .

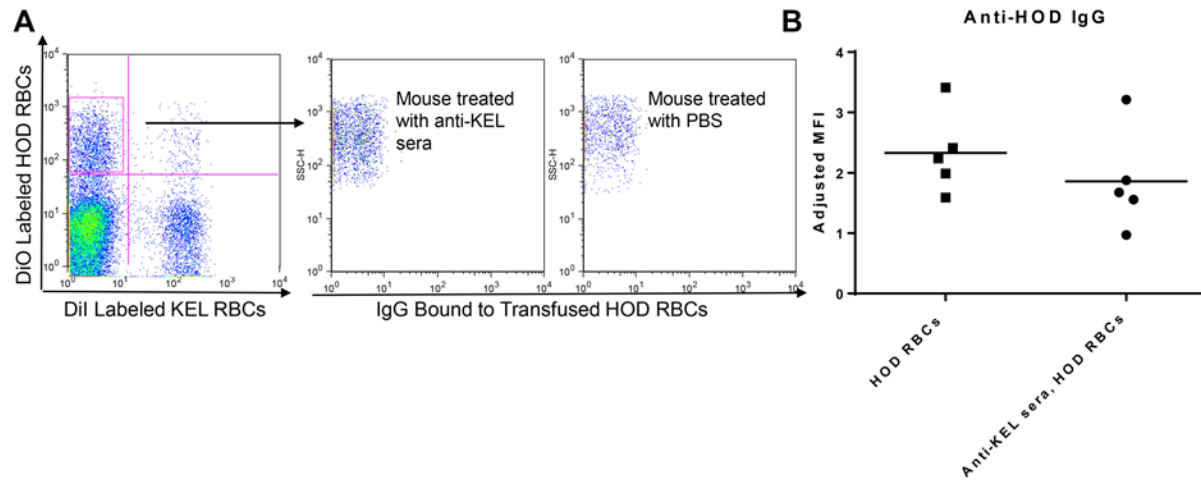
## **Study approval**

All procedures and protocols were approved by Emory and Yale University's Institutional Care and Use Committees.

**Supplementary Figure 1: Anti-KEL sera is passively administered and is tracked in recipients post-infusion by flow cytometry. (A)** In vitro titration of polyclonal anti-KEL crossmatched with murine KEL RBCs; solid grey line is sera at 1:5 dilution, bolded black line is sera at 1:50 dilution and that used for further described experiments, dashed line is sera at 1:5000 dilution, plain black line is sera at 1:50,000 dilution and shaded grey histogram is saline alone. **(B)** Mouse Ig staining (direct antiglobulin testing) on all recipient RBCs in non-transfused (left panel) or transfused recipients. **(C)** Measurement by flow cytometric crossmatching of KEL specific IgG over time in recipients passively infused with anti-KEL sera; adjusted MFI reflects the signal from recipient sera crossmatched with C57BL/6 RBCs subtracted from that of sera crossmatched with murine KEL RBCs. Data shown in C are representative of 3 independent experiments, with 3 mice/group/experiment.

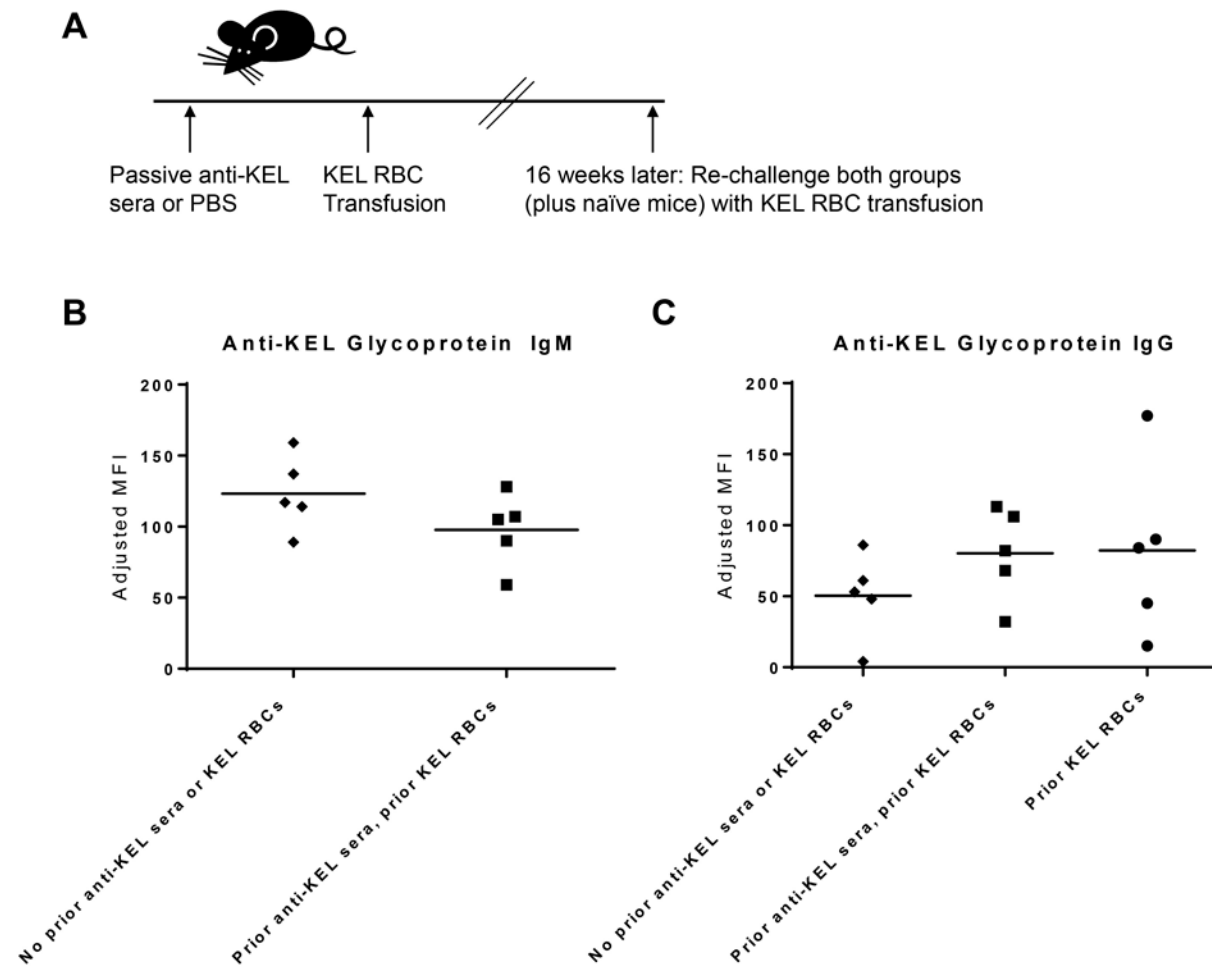


**Supplementary Figure 2: Anti-KEL sera prevents alloimmunization to KEL but not a 3<sup>rd</sup> party antigen.** (A) Representative flow plot from a transfusion recipient of DiO labeled HOD and DiI labeled KEL RBCs, with evaluation of bound IgG to transfused HOD RBCs. (B) Anti-HOD IgG responses in recipient serum, measured 2 weeks post-transfusion by flow cytometry. Data shown are representative of 2-3 experiments, with 3-5 mice/group/experiment.

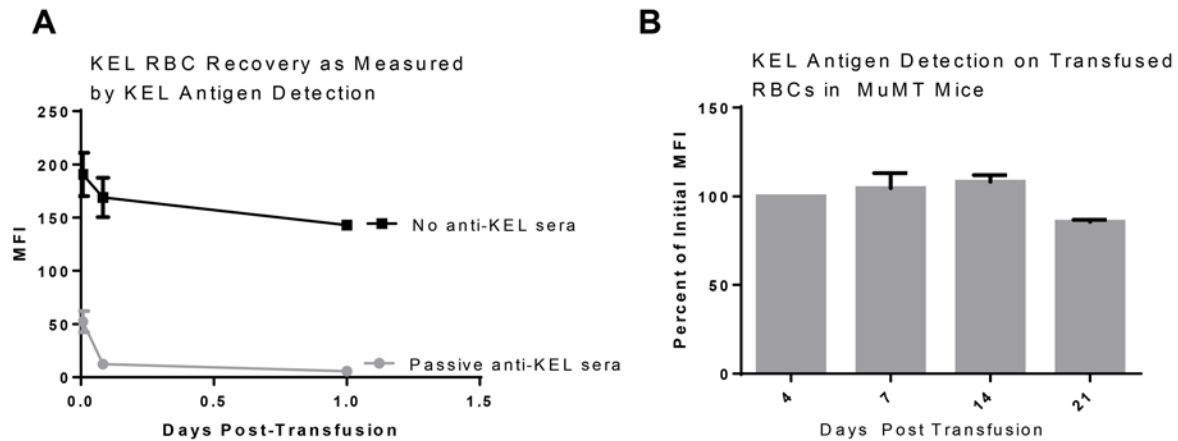




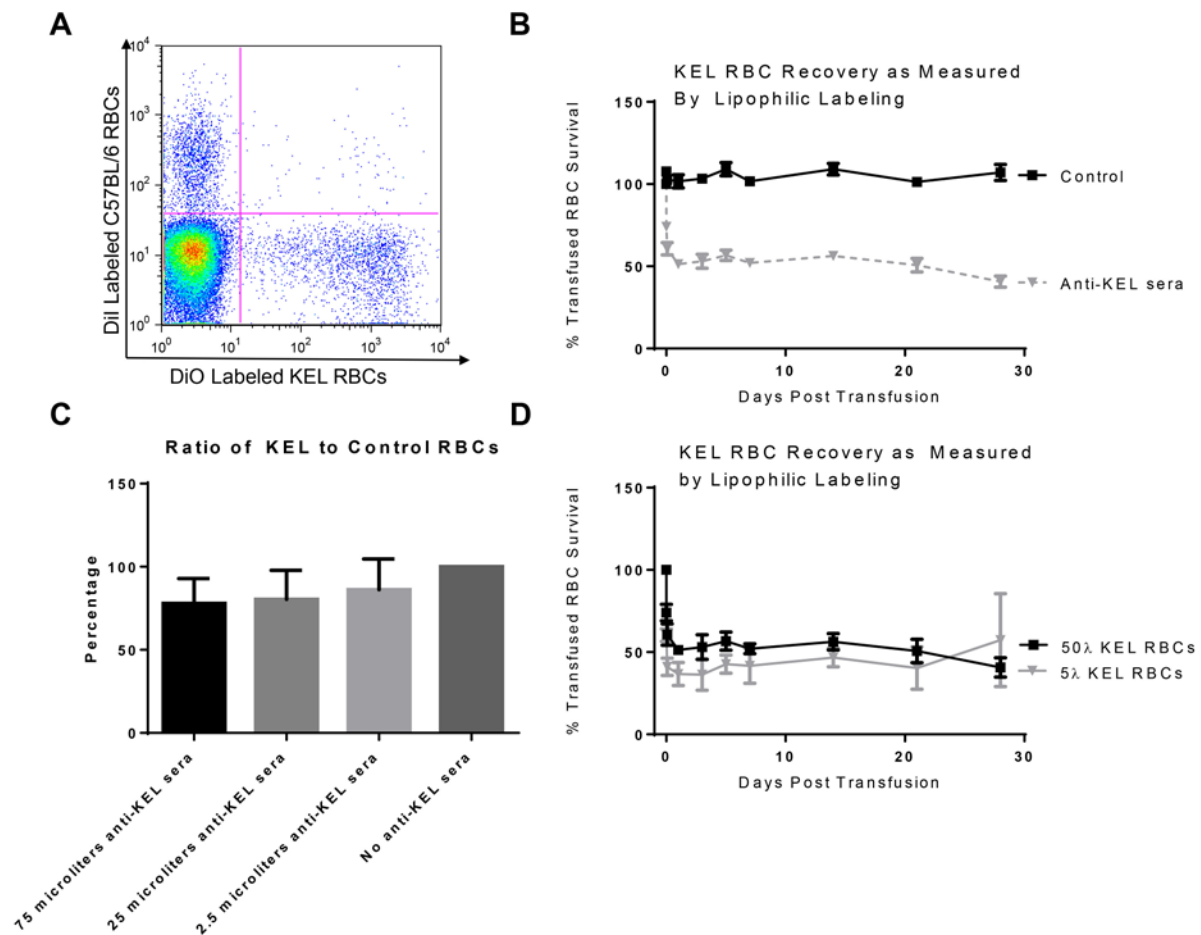
**Supplementary Figure 3: Prior therapy with anti-KEL sera does not lead to long term non-responsiveness or tolerance.** (A) Schematic of experimental design. (B) Recipient anti-KEL glycoprotein IgM responses in serum as detected by flow crossmatch 5 days post-transfusion, in naïve mice or in mice initially treated with anti-KEL sera and KEL RBCs 4 months prior. (C) Recipient anti-KEL glycoprotein IgG responses in serum as detected by flow crossmatch 28 days post-transfusion, in naïve mice, mice initially treated with anti-KEL sera and KEL RBCs 4 months prior, or mice initially transfused with KEL RBCs 4 months prior. Data shown are representative of 3 experiments, with 3-5 mice/group/experiment.



**Supplementary Figure 4: Transfused KEL RBCs appear to be rapidly cleared following transfusion into animals pretreated with anti-KEL sera, using a phenotypic RBC tracking approach. (A)** Detection of transfused KEL RBCs, using polyclonal anti-KEL to stain recipient blood at serial time points post-transfusion. **(B)** Evaluation of the stability of the KEL antigen on RBCs transfused into MuMT recipients, using polyclonal anti-KEL to stain recipient blood at serial time points post-transfusion. Data shown are representative of 3 independent experiments, with 3-5 mice/group/experiment. Error bars represent standard deviation.



**Supplementary Figure 5: Lipophilic labeling of KEL RBCs prior to transfusion shows pretreatment with anti-KEL sera does not clear all transfused RBCs.** (A) Representative flow plot from a transfusion recipient of lipophilically labeled KEL and control C57BL/6 RBCs. (B) KEL RBC post-transfusion recovery over time as determined using a ratio of lipophilically labeled KEL to control RBCs, in syngeneic (black line) or wild type (grey line) recipients. (C) KEL RBC recovery at 2 hours post-transfusion in recipients treated with titrated doses (2.5 to 75  $\mu$ L) of KEL Ig. (D) KEL RBC recovery over time in recipients treated with anti-KEL sera and then transfused with titrated doses (black line is 50 $\lambda$ , grey line is 5 $\lambda$ ) of KEL RBCs. Data shown in A and B are representative of 3 experiments with 3-5 mice/group/experiment (for B,  $p < 0.05$  at all studied time points); data shown in C and D are representative of 2 experiments with 3 mice/group/experiment. Error bars represent standard deviation



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