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Journal Title: Blood Advances
Volume: Volume 2, Number 2
Publisher: American Society of Hematology | 2018-01-23, Pages 105-115
Type of Work: Article | Final Publisher PDF
Permanent URL: https://pid.emory.edu/ark:/25593/s7q1k

Final published version:
http://www.bloodadvances.org/content/2/2/105/tab-article-info

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Accessed November 24, 2019 10:17 PM EST
Recipient priming to one RBC alloantigen directly enhances subsequent alloimmunization in mice

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Key Points

• CD4+ T cells primed to one RBC alloantigen promote humoral immunity to a disparate RBC alloantigen when both antigens are on the same RBC.

• These findings provide a potential explanation for how responses to one antigen may enhance antibody formation to other RBC alloantigens.

Individuals that become immunized to red blood cell (RBC) alloantigens can experience an increased rate of antibody formation to additional RBC alloantigens following subsequent transfusion. Despite this, how an immune response to one RBC immunogen may impact subsequent alloimmunization to a completely different RBC alloantigen remains unknown. Our studies demonstrate that Kell blood group antigen (KEL) RBC transfusion in the presence of inflammation induced by poly (I:C) (PIC) not only enhances anti-KEL antibody production through a CD4+ T-cell–dependent process but also directly facilitates anti-HOD antibody formation following subsequent exposure to the disparate HOD (hen egg lysozyme, ovalbumin, fused to human blood group antigen Duffy b) antigen. PIC/KEL priming of the anti-HOD antibody response required that RBCs express both the KEL and HOD antigens (HOD×KEL RBCs), as transfusion of HOD RBCs plus KEL RBCs or HOD RBCs alone failed to impact anti-HOD antibody formation in recipients previously primed with PIC/KEL. Transfer of CD4+ T cells from PIC/KEL-primed recipients directly facilitated anti-HOD antibody formation following (HOD×KEL) RBC transfusion. RBC alloantigen priming was not limited to PIC/KEL enhancement of anti-HOD alloantibody formation, as HOD-reactive CD4+ T cells enhanced anti-glycophorin A (anti-GPA) antibody formation in the absence of inflammation following transfusion of RBCs coexpressing GPA and HOD. These results demonstrate that immune priming to one RBC alloantigen can directly enhance a humoral response to a completely different RBC alloantigen, providing a potential explanation for why alloantibody responders may exhibit increased immune responsiveness to additional RBC alloantigens following subsequent transfusion.

Introduction

Chronic red blood cell (RBC) transfusion support is a vital therapy for patients with congenital hemoglobinopathies. Indeed, RBC transfusions can significantly reduce complications in these patients.1 However, one of the challenges in transfusion therapy is the development of alloantibodies to polymorphic RBC antigens, which appears to substantially increase the risk of developing additional alloantibodies to newly encountered RBC alloantigens in some patients.1,2 Patients that experience this long-recognized clinical phenomenon can experience a significant barrier to receiving compatible RBCs for future transfusions, which can directly contribute to increased morbidity and mortality in this transfusion-dependent population.4,5 Although antigen matching can reduce rates of alloimmunization, recent studies demonstrate that antigen-matching protocols can fail to prevent RBC alloimmunization.
and transfusion-associated negative consequences.6,7 However, why alloantibody formation against one alloantigen appears to increase the rate of alloimmunization against completely distinct RBC alloantigens remains a fundamental question in the field that has persisted for nearly 60 years. Several factors have been hypothesized to govern susceptibility to alloimmunization, including general differences in immune function and the potential impact of recipient inflammation at the time of transfusion.8-15 However, as an immune response to one RBC alloantigen correlates with an increased likelihood of antibody formation against a completely different alloantigen, it remains possible that the distinct immunological responses induced following exposure to certain RBC alloantigens may directly facilitate the development of additional alloantibodies following subsequent exposure to disparate RBC alloantigens.

Except for ABO(H), I and other carbohydrate blood group antigens, the vast majority of clinically relevant RBC antigens (eg, Kell, Kidd, and Duffy) are proteins or glycoproteins capable of eliciting antibody formation through a T-cell–dependent (TD) process. Consistent with this, CD4⁺ T cell peptides have been identified within certain RBC antigens,16,17 and HLA class II variants have been found to correlate with RBC alloimmunization,17-26 indicating a requirement for CD4⁺ T cell help. Moreover, studies using the murine RBC model antigen HOD, a fusion protein consisting of hen egg lysozyme, ovalbumin, and the human blood group antigen Duffy, recently demonstrated that anti-HOD antibody formation is likewise dependent on CD4⁺ T cells.27,28 Classically, CD4⁺ T cell help can occur through direct recognition of a peptide-major histocompatibility complex (MHC) complex that resides within or is directly linked to a target B-cell antigen.29,30 However, unlike the canonical pathways of T-cell help described above, individuals who develop alloantibodies to one RBC alloantigen appear to experience a direct enhancement of alloantibody formation against completely new RBC alloantigens following subsequent transfusion.1,3 These clinical observations suggest that CD4⁺ T cells specific to one RBC alloantigen may actually facilitate immunity to a completely different RBC alloantigen following subsequent exposure.

To study the potential ability of immunization to one RBC alloantigen to directly impact an immune response to a completely different RBC alloantigen following subsequent RBC exposure, we used 3 distinct yet well-characterized RBC alloimmunization mouse models that express the human KEL (Kell blood group antigen), model HOD, or human glycoporphin A (GPA) antigen on RBCs.27,28,31-38 Using these systems, we found that exposure to KEL in the presence of inflammation generates a CD4⁺ T-cell immune response that is capable of boosting a humoral response to the completely distinct HOD antigen. Furthermore, HOD reactive CD4⁺ T cells possess a similar ability to enhance anti-GPA antibody formation. These findings demonstrate that CD4⁺ T cells primed against one RBC antigen may facilitate the development of humoral immunity against a newly encountered RBC alloantigen and thereby suggest that the immune response to one RBC alloantigen can directly impact alloimmunization to different RBC alloantigens.

**Materials and methods**

**Mice**

Female B6 (C57BL/6) and MHC class II knockout (KO; B6.129S2-H2<sup>abI-Ea/J</sup>) recipients were purchased from Charles River Laboratories (Wilmington, MA) and The Jackson Laboratory (Bar Harbor, ME), respectively. GPA transgenic mice were maintained as outlined previously.38 KEL and HOD mice were generated as outlined previously.38-31 (HOD × KEL) F1 and (HOD × GPA) F1 donors were generated by breeding KEL and HOD mice or GPA and HOD mice.37,39 OTII (H-2<sup>K<sup>) donors were a gift from Jacob Kohlmeier (Emory University). All mice were used at 8 to 12 weeks of age, and were bred and housed in the Emory University Department of Animal Resources facilities. All procedures were performed according to approved Institutional Care and Use Committee protocols.

**RBC transfusion**

Indicated recipients were administered a single intraperitoneal injection of 100 µg poly (I:C) (PIC; GE Healthcare Bio-Sciences) 2 to 4 hours prior to transfusion, as done previously.36-40,43 Donor KEL, HOD, (HOD × KEL), GPA, or (HOD × GPA) blood was collected into acid citrate dextrose (Vacutainer) and washed with phosphate-buffered saline (PBS), and corresponding recipients were then transfused via the tail vein with 50 µL packed KEL, HOD, (HOD × KEL), a mixture of 50 µL HOD RBCs and 50 µL KEL RBCs, GPA, (HOD × GPA), or a mixture of HOD RBCs and GPA RBCs diluted in PBS to 300 µL total volume (equivalent to 1 human unit).

**Adoptive transfer**

20 × 10⁶ OTII splenocytes were adoptively transferred into indicated recipients via tail vein injection. For CD4⁺ T-cell adoptive transfer, CD4⁺ T cells from naive B6 donors or those transfused 2 times a week apart with KEL RBCs in the presence of PIC were negatively selected using a Miltenyi Biotec CD4⁺ T-cell isolation kit. 15 × 10⁶ CD4⁺ T cells were transferred into specified B6 recipients through tail vein injection.

**CD4⁺ T-cell depletion**

CD4⁺ T-cell depletion was achieved by 2 intraperitoneal injections of 250 µg monoclonal anti-mouse CD4 antibody (clone GK1.5, Bio X Cell) diluted in PBS 4 and 2 days prior to transfusion.44 Additional B6 recipients were similarly treated with 250 µg rat immunoglobulin G2b (IgG2b; clone LTF2, Bio X Cell) antibody diluted in PBS. Depletion efficacy was assessed prior to transfusion by staining peripheral blood leukocytes with fluorescein isothiocyanate anti-mouse CD3c, allophycocyanin (APC) anti-mouse CD4 (clone RM-45), and phycoerythrin anti-mouse CD8a (BD Bioscience). Samples were run on a BD FACScalibur and analyzed using FlowJo.

**Seroanalysis**

Serum collected 5 and 14 days posttransfusion was evaluated for anti-KEL, anti-HOD, and anti-GPA antibodies through indirect immunofluorescence staining.32-36,39-41,43,45-50 Neat serum was incubated with KEL, HOD, GPA, or B6 RBCs for 15 minutes at room temperature, washed with fluorescence-activated cell sorting (FACS) buffer (PBS + 2% bovine serum albumin + 0.9 g EDTA), and subsequently incubated for 30 minutes with APC anti-mouse IgG and fluorescein isothiocyanate anti-mouse IgM (Jackson ImmunoResearch) diluted 1:100 in FACS buffer. Samples were run on a BD FACScalibur and analyzed using FlowJo; mean fluorescence intensity (MFI) was used to measure serum anti-KEL, anti-HOD, or anti-GPA antibodies, and were calculated by normalizing the MFI of HOD RBCs, GPA RBCs or KEL RBCs to the MFI of background control B6 RBCs.
Statistics

Statistical analysis was performed using GraphPad software and 1-way analysis of variance (ANOVA) with Tukey’s post-test or Student t test. Significance was determined by P < .05.

Results

Inflammation induced by PIC facilitates a TD antibody response to KEL RBCs

As the HOD system represents the only known murine RBC alloimmunization model capable of inducing a TD alloantibody response in the absence or presence of inflammation,27,42 HOD RBCs were used as a well-characterized secondary RBC antigen exposure. As there is currently no other murine RBC antigen model capable of inducing a TD antibody response, a distinct murine model RBC antigen capable of inducing a CD4+ T cell response was developed. To date, the primary alternative system to HOD known to recapitulate the clinical features of RBC alloimmunity (ie, antibody formation, RBC clearance, hemolytic disease of the fetus and newborn)23,24 is the KEL transgenic system, which expresses Kell (KEL) specifically on RBCs.31 In contrast to HOD, KEL RBCs induce anti-KEL antibodies independent of CD4+ T cells,43 possibly preventing the KEL system from providing the T-cell priming needed to impact subsequent TD HOD RBC exposure. However, recipient exposure to PIC, a double-stranded RNA that induces inflammation, can enhance alloimmunization to KEL RBCs35 and facilitate CD4+ T-cell activation to the model RBC antigen hen egg lysozyme,42 suggesting that PIC may enhance KEL alloimmunization through T-cell help.

To test this, CD4− T-cell–depleted B6 recipients (Figure 1A) were transfused with KEL RBCs in the presence or absence of PIC. Consistent with previous reports,30,41 PIC treatment enhanced the anti-KEL IgG response (Figure 1B). However, CD4− T-cell depletion resulted in comparable anti-KEL IgG regardless of PIC exposure (Figure 1B). The lack of a detectable increase in anti-KEL IgG in PIC-treated recipients depleted of CD4+ T cells was not due to nonspecific effects of the antibody itself, as isotype control antibody–treated recipients experienced a PIC-enhanced anti-KEL IgG response (Figure 1B). It is as possible that the CD4-depleting antibody concomitantly depleted other CD4+ leukocytes that may boost alloimmunity to KEL, the ability of PIC to enhance KEL alloimmunization was next examined in MHC II KO recipients that possess significantly reduced CD4+ T cells (Figure 1A). Analogous to CD4− T-cell depletion, PIC-treated MHC II KO recipients did not generate a boosted anti-KEL IgG response (Figure 1C). Together, these findings suggest that PIC treatment enhances KEL alloimmunization through a CD4+ T cell process.

HOD alloimmunization is boosted in PIC/KEL-primed recipients following (HOD × KEL) RBC transfusion

As PIC facilitated KEL alloimmunization (PIC/KEL) in a CD4+ TD manner, we next sought to examine the potential impact of KEL reactive CD4+ T-cell immunity on an alloantibody response to HOD following subsequent transfusion of RBCs that express both the HOD and KEL antigens. To test this, HOD and KEL mice were crossed to generate (HOD × KEL) progeny, providing an opportunity to examine the impact of subsequent (HOD × KEL) antigen exposure following PIC/KEL priming (Figure 2A). PIC/KEL-primed recipients developed an anti-HOD IgM response that was statistically comparable to that of naive recipients following (HOD × KEL) RBC transfusion (Figure 2B). However, in contrast to the anti-HOD antibody response detected in naive recipients transfused with (HOD × KEL) RBCs, prior PIC/KEL priming significantly enhanced the anti-HOD IgG response following (HOD × KEL) RBC transfusion (Figure 2B). Naive recipients exposed to HOD RBCs, (HOD × KEL) RBCs, or (HOD + KEL) RBCs exhibited an equivalent anti-HOD antibody response. Furthermore, PIC/KEL-primed recipients transfused with HOD RBCs alone or (HOD + KEL) RBCs did not demonstrate increased anti-HOD IgG (Figure 2B). Overall, these results suggest that PIC/KEL priming can facilitate humoral immunity against the completely distinct HOD antigen when HOD and KEL are coexpressed on subsequently transfused RBCs.

Immunity to KEL in the absence of PIC does not boost an antibody response to HOD

The ability of PIC/KEL priming to boost HOD following (HOD × KEL) RBC transfusion, coupled with the ability of PIC to enhance KEL alloimmunization in a TD manner, strongly suggests that PIC drives a KEL reactive CD4+ T-cell response that subsequently facilitates anti-HOD antibody formation following (HOD × KEL) RBC transfusion. To determine whether PIC is necessary to induce a KEL alloimmune response capable of priming HOD alloimmunization, B6 recipients were primed against KEL in the absence of PIC and subsequently transfused with (HOD × KEL) RBCs. In contrast to the impact of PIC/KEL priming (Figure 2), recipients primed with KEL RBCs in the absence of PIC failed to demonstrate a detectable increase in anti-HOD antibodies following subsequent (HOD × KEL) RBC transfusion when compared with (HOD × KEL) RBC transfusion alone (Figure 3A). Similar results were observed post transfusion of HOD RBCs or (HOD + KEL) RBCs (Figure 3A). The inability to boost HOD in KEL-sensitized recipients was not due to the lack of KEL alloimmunization, as anti-KEL IgG was detectable in all recipients primed with KEL RBCs (Figure 3B). Although these results do not exclude the possibility that KEL RBCs can activate CD4+ T cells in the absence of PIC and that these CD4+ T cells could contribute to KEL alloimmunization when present, they are consistent with the ability of KEL RBCs to induce anti-KEL antibodies independent of CD4+ T cells. Moreover, these results suggest that whatever CD4+ T-cell activation occurs following KEL RBC transfusion appears to be insufficient to impact not only KEL alloimmunization but also the antibody response to HOD following (HOD × KEL) RBC transfusion. Taken together, these results suggest that while KEL priming in the presence of PIC can boost a KEL immune response in a CD4+ TD manner and similarly enhance HOD immunity following subsequent (HOD × KEL) RBC transfusion, recipient priming with intrinsically CD4+ T-cell–independent (TII) KEL RBCs alone fails to impact subsequent HOD alloimmunization.

PIC treatment increased KEL alloimmunization through a TD process (Figure 1) and HOD is a TD antigen,27 suggesting that KEL-reactive CD4+ T cells prime humoral immunity to HOD when HOD and KEL are expressed on the same RBCs. To examine whether this is specific to a TD immune response, we next examined whether HOD-specific CD4+ T cells can boost alloimmunity to the KEL antigen that can generate an antibody response independent of T cells.43 To test this, B6 recipients were adoptively transfused with OTII splenocytes from a T-cell receptor transgenic that specifically recognizes ovalbumin within the HOD antigen25; adoptive transfer of OTII significantly augments the anti-HOD antibody response. Following transfer, recipients were transfused with (HOD × KEL) RBCs. Consistent
with the ability of KEL RBCs to induce a robust TI antibody response, B6 recipients transfused with (HOD $\times$ KEL) RBCs in the presence or absence of OTIIIs generated an anti-KEL antibody response that was comparable to recipients transfused with KEL RBCs or (HOD $\times$ KEL) RBCs (Figure 4A). The failure to boost an anti-KEL IgG response was not due to the lack of a sufficient alloimmune response to HOD, as recipients adoptively transferred with OTIIIs experienced a significant boost in anti-HOD IgG following HOD exposure (Figure 4B). However, to determine whether primed HOD-reactive CD4$^+$ T cells can augment an anti-KEL antibody response following (HOD $\times$ KEL) RBC transfusion, B6 recipients were transfused with HOD RBCs, followed by a similar secondary (HOD $\times$ KEL) RBC exposure. Similar to OTII adoptive transfer, prior exposure to HOD RBCs failed to significantly impact the level of anti-KEL antibody

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Figure 1. PIC enhances KEL alloimmunization through a CD4$^+$ TD process. (A) Representative gating strategy and graphical demonstration of the percentage of splenic CD3$^+$ CD4$^+$ T cells in B6 recipients treated with PBS (B6), anti-mouse CD4-depleting antibody (CD4 depl.), or rat IgG2b isotype control antibody (Iso. Cont.) and CD4$^+$ T-cell–deficient MHC II KO mice. (B) Anti-KEL IgG formation in recipients treated with PBS (B6), anti-mouse CD4-depleting antibody (CD4 depl.), and rat IgG2b isotype control antibody (Iso. Cont.) and transfused with KEL RBCs in the presence or absence of 100 $\mu$g PIC. (C) MHC Class II KO (MHC II KO) recipients transfused with KEL RBCs in the presence or absence of 100 $\mu$g PIC. Serum was collected on day 14 (D14) after transfusion, and serological analysis for anti-KEL IgG alloantibodies in panels B-C was done by indirect immunofluorescence staining using KEL and B6 RBCs. The MFI in panels B-C was derived from normalizing the MFI of serum samples incubated with KEL RBCs to the MFI of serum samples incubated with background control B6 RBCs. Error bars represent mean ± standard error of the mean (SEM). The mean of each group is depicted as a horizontal line. Statistics were generated using a 1-way ANOVA with a Tukey's post-test in panels A-B and a Student t test in panel C. Data in panel A are representative of 3 experiments with 3 to 5 mice per group. Panels B-C show a combination of data from 2 to 3 experiments with 4 to 6 mice per group. **$P < .01$; ***$P < .001$; ****$P < .0001$; n.s., nonsignificant. FSC, forward scatter; SSC, side scatter.
formation following subsequent (HOD × KEL) RBC transfusion (supplemental Figure 1).

**PIC/KEL-primed CD4^+ T cells alone enhance HOD alloimmunity after (HOD × KEL) RBC exposure**

The findings thus far suggest that KEL-reactive CD4^+ T cells can boost humoral immunity to the disparate antigen HOD following (HOD × KEL) RBC transfusion. However, to directly test whether KEL-reactive CD4^+ T cells can enhance immunity to HOD, CD4^+ T cells from naive and PIC/KEL-primed recipients were enriched and adoptively transferred into naive B6 recipients that were subsequently transfused with (HOD × KEL) RBCs (Figure 5A). Recipients adoptively transferred with PIC/KEL-primed CD4^+ T cells were found to mediate a statistically significant boost in anti-HOD IgG following (HOD × KEL) RBC exposure (Figure 5B). The boosted anti-HOD IgG response was specific to the presence of PIC/KEL-primed CD4^+ T cells, as adoptive transfer of naive CD4^+ T cells failed to generate the same degree of an anti-HOD antibody response despite coexpression of KEL on (HOD × KEL) RBCs (Figure 5B). Likewise, recipients adoptively transferred with naive or PIC/KEL-primed CD4^+ T cells failed to generate an enhanced anti-HOD IgG response following HOD RBC exposure (Figure 5B).

Combined, these findings demonstrate that PIC/KEL-primed CD4^+ T cells can provide sufficient help to the completely distinct HOD antigen following subsequent (HOD × KEL) RBC transfusion.

**CD4^+ T-cell immunity to HOD induces an antibody response to GPA following (HOD × GPA) RBC transfusion**

Despite the ability of adoptively transferred CD4^+ T cells isolated from PIC/KEL-primed recipients to facilitate an anti-HOD antibody response following (HOD × KEL) RBC transfusion, it is possible that PIC itself may artificially prime CD4^+ T cells. To determine whether CD4^+ T cells can enhance a humoral response to an unrelated antigen in the absence of PIC, we next turned to the GPA model.38 Similar to the lower antibody response observed following HOD RBC transfusion alone, in the absence of inflammation, transfusion of GPA actually fails to induce detectable increases in anti-GPA antibody formation.36,52 Thus, to determine whether CD4^+ T cells against HOD may alter GPA responsiveness following GPA RBC exposure, recipients were adoptively transferred with OTIIs in the absence of PIC and subsequently transfused with HOD RBCs, GPA RBCs, (HOD × GPA) RBCs that express the HOD and GPA antigens on the same RBCs,37 or a mixture of HOD RBCs and GPA.
RBCs (HOD + GPA RBCs). Consistent with previous findings, recipients exposed to GPA RBCs alone or (HOD + GPA) RBCs failed to induce a significant antibody response to GPA in the presence or absence of OTIIs (Figure 6). Similarly, transfusion of (HOD × GPA) RBCs in the absence of OTIIs did not induce a detectable antibody response to GPA (Figure 6). However, recipients adoptively transferred with OTIIs induced a statistically significant IgM and IgG response to GPA following secondary exposure to (HOD × GPA) RBCs (Figure 6). Together, these findings demonstrate that CD4+ T-cell immunity to one RBC alloantigen can induce an antibody response to a completely distinct RBC alloantigen.

**Discussion**

The ability of CD4+ T cells sensitized to one RBC alloantigen to directly augment a humoral response to a disparate RBC alloantigen may in part explain the apparent propensity of some responders to exhibit increased immune responsiveness to additional RBC antigens.

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**Figure 3.** Recipient priming with KEL in the absence of inflammation does not boost the HOD antibody response. Where indicated, B6 recipients were primed against KEL prior to transfusion of HOD RBCs, KEL RBCs, (HOD × KEL) RBCs, or HOD RBCs mixed with KEL RBCs (HOD + KEL) 14 days later. (A-B) Anti-HOD IgM (A), anti-HOD IgG (A), and anti-KEL IgG (B) alloantibodies were examined at days 5 (D5 for IgM) and 14 (D14 for IgG) after transfusion using an indirect immunofluorescence staining using HOD, KEL, and B6 RBCs. The MFIs in panels A-B were calculated by normalizing the MFI of HOD RBCs (A) or KEL RBCs (B) to the MFI of background control B6 RBCs. Error bars represent mean ± SEM. The mean of each group is depicted as a horizontal line. Statistics were generated using a 1-way ANOVA with a Tukey’s post-test. All panels show combinatorial data from experiments reproduced 2 times, with each repeat consisting of 5 mice per group. *P < .05; **P < .01; ****P < .0001.

**Figure 4.** CD4+ T cells reactive to HOD do not boost an antibody response to KEL. Indicated B6 recipients were adoptively transferred with OTII splenocytes that were enriched in CD4+ T cells specific for the HOD antigen. 24 hours following adoptive transfer, all recipients were transfused with HOD RBCs, KEL RBCs, (HOD × KEL) RBCs, or a mixture of HOD RBCs and KEL RBCs (HOD + KEL). (A-B) Anti-KEL IgM (A), anti-KEL IgG (A), and anti-HOD IgG (B) antibody formation is shown for transfused recipients 5 days (D5 for IgM) and 14 days (D14 for IgG) after transfusion. Serological analysis for anti-KEL or anti-HOD alloantibodies was done by indirect immunofluorescence using KEL and HOD RBCs. The MFI depicted was generated by normalizing the MFI of serum samples incubated with KEL (A) or HOD (B) RBCs to the MFI of serum samples incubated with background control B6 RBCs. Error bars represent mean ± SEM. The mean of each group is depicted as a horizontal line. Statistics were generated using a 1-way ANOVA with a Tukey’s post-test. All panels show combinatorial data from experiments reproduced 2 or 3 times, with 5 mice per group. ****P < .0001.
following subsequent transfusion. Interestingly, the ability of allor- reactive CD4$^+$ T cells to facilitate alloantibody production to a nontarget RBC antigen only occurred when recipients were first immunized against KEL and not HOD; prior transfusion of HOD RBCs or transfer of CD4$^+$ T cells specific to HOD was unable to significantly alter humoral immunity to KEL following (HOD × KEL) RBC exposure. However, as humoral immunity to KEL RBCs can occur independent of T cells, it is likely that the failure of HOD reactive CD4$^+$ T cells to boost an antibody response to KEL on (HOD × KEL) RBCs is due to the ability of KEL to induce a TI humoral response. Furthermore, the ability of OTII to induce a humoral response to GPA following (HOD × GPA) transfusion demonstrates that these CD4$^+$ T cells are capable of enhancing an antibody response toward an unrelated RBC antigen. Taken together, these findings indicate that fundamental features of RBC alloimmunization may be driven by the unique characteristics of each RBC antigen and indicate that the order of RBC alloantigen exposure may dictate the immunological outcome to a disparate RBC antigen during future transfusion events.

Given that several RBC alloantigens appear to be HLA restricted$^{17-26}$ and CD4$^+$ T cell peptides have been identified to certain RBC antigens,$^{16,17}$ the ability of KEL RBCs to induce a CD4$^+$ TI antibody response is indeed unexpected. Traditionally, TI immunogens are thought to be mitogenic stimuli that can activate B cells via ligation of pattern recognition receptors (TI type I antigens)$^{53}$ or extensive cross-linking of B-cell receptors by carbohydrate antigens with highly organized, repetitive structures (TI type II antigens).$^{54}$ In contrast, the factors that govern the ability of KEL to induce antibodies in the absence of CD4$^+$ T cells remain unknown. However, as recent studies suggest that the antigen density of KEL can impact immune outcomes following KEL exposure,$^{39}$ distinct RBC alloantigen densities may indeed impact the type of immune pathways that are engaged following RBC alloantigen exposure.

As inflammation enhanced KEL alloimmunization through a TD response and a boostable antibody response to HOD was only observed in PIC/KEL primed recipients, the inflammatory status of a recipient at the time of an initial priming event may influence the risk of generating immunity to disparate RBC alloantigens. The effect of a recipient’s inflammatory status on alloimmunization to a RBC antigen has been investigated clinically and in models. Previous clinical data demonstrate that the inflammatory events in sickle cell disease patients appear to increase the likelihood of RBC alloimmunization.$^8,9,55-58$ Similarly, murine recipients transfused in the presence of inflammation demonstrate higher magnitudes of RBC alloimmunization.$^{36,40-42}$ As inflammation was necessary for immunity to KEL to enhance antibody formation to HOD, it is possible that inflammation itself may prime CD4$^+$ T cells to facilitate the development of an antibody response to HOD following (HOD × KEL) RBC transfusion. However, in the absence of known inflammation, HOD-reactive CD4$^+$ T cells rendered recipients responsive to GPA following transfusion of (HOD × GPA) RBCs. These findings in no way rule out the possibility that when present, recipient inflammation may not only influence the primary immunizing event but also directly impact the immunological outcome of additional RBC alloantigens. Rather, these findings indicate that CD4$^+$ T cells reactive one RBC alloantigen can directly facilitate antibody formation against newly encountered RBC alloantigens in

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**Figure 5.** Transfer of CD4$^+$ T cells from PIC/KEL-primed donors enhances HOD alloimmunization following exposure to (HOD × KEL) RBCs. (A) Representative gating strategy of CD4$^+$ T-cell enrichment. CD4$^+$ T cells enriched from naive or PIC/KEL-primed B6 recipients were adoptively transferred 24 hours prior to transfusion of HOD RBCs alone or (HOD × KEL) RBCs. Anti-HOD IgG (B) was evaluated 14 days (D14) after transfusion by indirect immunofluorescence staining using HOD and B6 RBC targets. The MFI in panel B was derived from normalizing the MFI of HOD RBCs to the MFI of background control B6 RBCs. Error bars represent mean ± SEM. The mean of each group is depicted as a horizontal line. Statistics were generated using a 1-way ANOVA with a Tukey’s post-test. Panel B shows combinatorial data from 2 experiments, with at least 5 mice per group. *P < .05; ****P < .0001.
the absence of inflammation. Thus, based on our data, we propose that although the inflammatory milieu of an individual at the time of a primary immunizing event to a RBC alloantigen may influence immunity to that target antigen and subsequent alloimmunization, this type of environmental trigger during the priming event may not be required for all alloantigens.

The capacity of KEL- or HOD-reactive CD4⁺ T cells to promote a humoral response to HOD or GPA, respectively, indicates that cellular adaptive immunity to one RBC antigen may be responsible for enhancing an antibody response to a newly encountered RBC alloantigen. However, the number of transfusions a patient receives, genetic features of an individual's immune system, and/or environmental factors may additionally or independently govern susceptibility to alloimmune responses. Certainly, clinical data illustrate a general increasing trend in alloantibodies with each additional transfusion. However, a retrospective study examining factors that may impact alloimmunization incidence suggests that the prospect of repeat alloantibody formation weakly correlates with the number of transfusions a patient receives. The ability of individuals who experience an increased propensity to become immunized to additional RBC alloantigens may simply reflect underlying genetic and/or environmental factors that influence the likelihood of an alloimmune response. However, although HLA class II variants correlate with alloimmunization to a primary immunizing event to one RBC antigen, these associations are often not sufficient to increase the probability of developing a humoral response to newly encountered RBC antigens. Our findings do not rule out the possibility that when present, a variety of factors may influence the development of alloimmunization, including prior alloimmunization events, HLA and non-HLA variant genetics, and additional immune factors that may increase the susceptibility of developing alloimmunity to RBC antigens. However, as our murine model specifically examined the presence or absence of an established CD4⁺ T-cell response, the data presented herein strongly suggest that cellular adaptive immunity to one RBC antigen can sensitize against a subsequent alloantigen given that future transfusion products express both target alloantigens.

Patients with alloantibodies to RBC antigens typically receive compatible transfusion products to avoid hemolytic transfusion reactions, suggesting that our findings may not be clinically relevant. However, as the lack of detectable alloantibodies following a primary exposure does not necessarily reflect the absence of primed T cells, CD4⁺ T cells primed against one RBC antigen in the absence of detectable alloantibodies may facilitate humoral immunity to a completely disparate RBC immunogen during a future transfusion. Consistent with this, our data demonstrate that transfer of KEL-reactive CD4⁺ T cells into nonalloimmunized recipients boosted an anti-HOD alloantibody response following (HOD × KEL) RBC transfusion, and transfer of HOD-reactive CD4⁺ T cells likewise generated an anti-GPA antibody response following secondary exposure to (HOD × GPA) RBCs. Moreover, as RBCs express a variety of possible polymorphic antigens capable of inducing cellular adaptive immunity in the presence or absence of detectable antibodies secondary to their location inside the cell or other key biochemical features, it is conceivable that cellular adaptive immunity to these polymorphic antigens that may not be detected serologically can similarly influence the immunological consequence of RBC alloantigen exposure. However, whether immunization to such antigens correlates with susceptibility of becoming alloimmunized to clinically relevant RBC antigens necessitates further investigation. Currently, no clinical methodologies exist to screen for cellular adaptive immunity to many RBC alloantigens, making it difficult to ascertain this possibility. As a result, alloimmunization may be accompanied by undetected CD4⁺

![Figure 6. HOD reactive CD4⁺ T cells render recipients responsive to GPA following (HOD × GPA) RBC exposure.](image)

Where indicated, B6 recipients were adoptively transferred with OTII splenocytes. 24 hours following adoptive transfer, all recipients were transfused with HOD RBCs, GPA RBCs, (HOD × GPA) RBCs, or a mixture of HOD RBCs and GPA RBCs (HOD + GPA). (A-B) Anti-GPA IgM (A) and anti-GPA IgG (B) antibody formation is shown for transfused recipients 5 days (D5 for IgM) and 14 days (D14 for IgG) after transfusion. Serological analysis for anti-GPA alloantibodies was examined by indirect immunofluorescence staining using GPA and B6 RBCs. The MFI depicted was generated by normalizing the MFI of serum samples incubated with GPA RBCs to the MFI of serum samples incubated with background control B6 RBCs. Error bars represent mean ± SEM. The mean of each group is depicted as a horizontal line. Statistics were generated using a 1-way ANOVA with a Tukey’s post-test. All panels show combinatorial data from experiments reproduced 2 times, with 5 mice per group. ***P < .001; ****P < .0001.
T-cell responses that occur following prior transfusion events that facilitate subsequent immunity detected serologically.

Current concepts in TD antibody responses suggest that T cells facilitate humoral responses through 2 distinct pathways. Classically, CD4\(^+\) T-cell help can occur through direct recognition of a peptide–MHC complex comprising of an epitope on the target protein antigen that is bound by the B-cell receptor. Alternatively, CD4\(^+\) T cells can activate a naive B cell through a process termed “linked recognition,” where a CD4\(^+\) T-helper cell recognizes a peptide–MHC complex consisting of an epitope that resides within a protein immunogen that is linked to a dissimilar antigen detected by the B-cell receptor.\(^{39,62,63}\) Several studies utilizing “hapten-carrier” systems or viral models such as influenza demonstrate that in this scenario, the B cell and CD4\(^+\) T cell do not need to recognize identical epitopes on the same antigen, but rather, the peptides that drive T-cell help are typically physically linked to the conjugate recognized by the B cell.\(^{29,30,62,63}\) However, our results demonstrate that existing immunity to one RBC alloantigen can sensitize a recipient to an entirely distinct RBC immunogen following exposure to RBCs expressing both alloantigens, consistent with the clinical phenomenon of alloantibody responders. Thus, unlike with conjugate antigens, where the CD4\(^+\) T-cell peptide is directly derived from a protein immunogen conjugated to the B-cell epitope as part of a conjugate vaccine, virion, or virion-like particle, the pathway of CD4\(^+\) T-cell help proposed in the current study suggests that B cells possess the capacity to process 2 distinct antigens from a cell source roughly the same size as the B cell itself. This in turn appears to facilitate antibody formation following subsequent transfusion when a recipient is previously primed against one of the antigens.

Although this study focuses on the model antigens HOD, human GPA, and KEL, it is conceivable that this CD4\(^+\) T-cell priming may promote formation of alloantibodies to other RBC antigens and that these model antigens may simply reflect general principles that govern entirely different alloantigens altogether. This is especially important when considering that KEL-reactive CD4\(^+\) T cells can be identified in KEL-alloimmunized patients,\(^{16}\) suggesting that T-cell help may be important during a KEL immune response. However, as the events surrounding KEL priming in these studies remain unknown, these earlier clinical findings are also consistent with the possibility that recipient inflammatory events surrounding KEL priming may have facilitated the CD4\(^+\) T-cell response to KEL, and therefore do not exclude the possibility that KEL may induce alloantibody formation in the absence of T-cell help. As these findings are in a murine model, testing these hypotheses in a human setting will certainly be required before any clinical conclusions can be drawn. However, as not all known noncarbohydrate blood group and other polymorphic minor antigens on transfused RBCs elicit a T-cell response, which of these antigens is capable of activating CD4\(^+\) T cells would first need to be identified. Indeed, CD4\(^+\) T-cell peptides to certain RBC alloantigens (ie, RhD and Kell) have been identified in patients alloimmunized to that target RBC antigen,\(^{16,17}\) implicating a role of CD4\(^+\) T cells in alloantibody formation to RBC antigens in general.\(^{27,28,42,64,65}\) However, to the best of our knowledge, whether CD4\(^+\) T-cell immunity to one RBC alloantigen correlates with patient responsiveness to a disparate RBC alloantigen has not been investigated. Moreover, in the event that these antigens possess the ability to induce cellular adaptive immunity, the exact peptides required to generate a CD4\(^+\) T-cell response may significantly vary depending on the HLA variant of a given patient, and thereby would require elucidation on an individual basis. In addition, even if the CD4\(^+\) T-cell peptides for blood group and other minor antigens were identified, intentional primary and secondary exposure to RBCs for the purpose of experimentally inducing alloimmunization to multiple RBC alloantigens is unethical due to the potential risk of causing hemolytic transfusion reactions when only ABO- and RhD-compatible blood is provided emergently. Thus, prospectively testing the ability of CD4\(^+\) T-cell priming to one antigen to promote formation of alloantibodies to disparate RBC antigens in a clinical setting may be difficult. Nonetheless, these findings suggest important insight into an essential question that has persisted in the transfusion medicine field for over 60 years regarding alloantibody responders by providing potential insight into how individuals who respond to one RBC alloantigen may become susceptible to developing additional alloantibodies to other RBC alloantigens.

**Acknowledgments**

This work was supported in part by the Burroughs Wellcome Trust Career Award for Medical Scientists, the National Institutes of Health (NIH), National Heart, Lung, and Blood Institute (grant R01 HL135575), and Early Independence grant DP5OD019892 from the NIH Office of the Director (S.R.S.).

**Authorship**

Contribution: S.R.S. and S.R.P. designed the research study; S.R.P. carried out and analyzed experiments together with A.B., K.G.-P., C.L.M., S.C., and C.M.A.; P.E.Z. and A.M. provided critical support; and S.R.P. and S.R.S. wrote the manuscript, which was additionally edited and commented on by the other authors.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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