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Alloimmunization against RBC or PLT Antigens Is Independent of TRIM21 Expression in a Murine Model

Seema R Patel¹, Jeanne E Hendrickson^{2,1}, Nicole H Smith¹, Chantel M Cadwell¹, Keiko Ozato⁴, Herbert C Morse III³, Ryusuke Yoshimi⁴, and James C Zimring^{1,2,*}

¹ Center for Transfusion and Cellular Therapies, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA

² Aflac Cancer Center and Blood Disorders Service, Children's Healthcare of Atlanta, Division of Pediatric Hematology/Oncology, Emory University School of Medicine, Atlanta, GA

³ NIAID, NIH, Rockville, MD

⁴ NICHD, NIH, Bethesda, MD

Abstract

Generation of alloantibodies to transfused RBCs can be a serious medical problem for patients who require chronic RBC transfusion therapy. Patients with sickle cell disease have a substantially increased rate of alloimmunization compared to other chronically transfused populations. A recent study has forwarded the hypothesis that a polymorphism in an immunoregulatory gene in close proximity to beta-globin (TRIM21 rs660) plays a role in the increased rates of RBC alloimmunization in sickle cell patients. In particular, it was hypothesized that rs660C/T decreases expression of TRIM21, resulting in loss of a negative feedback pathway in immune responses and increased RBC alloimmunization. To test the effects of TRIM21 expression on alloimmunization, we analyzed antibody responses to alloantigens on RBCs and platelets transfused into wild-type and TRIM21 KO mice. No significant increases were seen in the frequency or magnitude of humoral immunization to alloantigens on transfused RBCs or platelets in adult or juvenile TRIM 21 KO recipients compared to wild-type controls. Moreover, recipient inflammation with poly (I:C) enhanced RBC alloimmunization to similar degrees in both TRIM 21 KO and wild-type control recipients. Together, these data rule out the hypothesis that decreased TRIM 21 expression enhances transfusion induced humoral alloimmunization, in the context of a reductionist murine model.

Keywords

Transfusion; alloimmunization; TRIM21; red blood cell; platelet

*To whom all correspondence should be addressed James Zimring, MD, Department of Pathology and Laboratory Medicine and Aflac Cancer Center and Blood Disorders Service, Center for Transfusion and Cellular Therapies, Emory University School of Medicine, Woodruff Memorial Building Suite 7107A, 101 Woodruff Circle, Atlanta, GA 30322, USA (Telephone 404-712-2174, Fax 404-727-5764) jzimrin@emory.edu.

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1. Introduction

Although RBC transfusion can be lifesaving, it is not without risk. One such risk includes the development of antibodies against foreign antigens on transfused donor RBCs (RBC alloimmunization). When a recipient mounts an antibody response against a given RBC antigen, subsequent transfusion with RBCs expressing the same antigen is typically contraindicated due to the risk of hemolysis of the transfused RBCs. Besides leading to costly and time consuming evaluations in the blood bank, RBC alloantibodies also put a patient at risk of delayed hemolytic transfusion reactions. Furthermore, RBC alloimmunization may also lead to hemolytic disease of the fetus and newborn in women of childbearing age. Thus, RBC alloimmunization is a significant hazard of transfusion.

In the general population of transfusion recipients, rates of RBC alloimmunization approximate 3%. However, certain patient populations have substantially higher rates; most notably, those with sickle cell disease (SCD). Overall, the RBC alloimmunization rate in SCD patients is approximately 25% (Garratty, 1997), with a range of reported rates between 8 and 47% (Aygun et al., 2002; Garratty, 1997; Luban, 1989; Orlina et al., 1978; Rosse et al., 1990; Tatari-Calderone et al., 2009; Vichinsky et al., 1990). This high rate of alloimmunization can lead to substantial difficulty and cost in identifying sufficient units of RBCs compatible for the needs of SCD patients, and in some cases, can lead to significant morbidity and/or mortality due to difficulty or inability to locate compatible units of RBCs.

The underlying mechanisms responsible for increased RBC alloimmunization in SCD patients remain unclear. It has been argued that increased rates of transfusion are responsible, as many SCD patients receive chronic transfusion for years. Furthermore, it has been argued that SCD patients encounter more foreign antigens than other groups, due to donor/recipient disparities in ethnic origin. The majority of blood donors in the United States are Caucasian, whereas the majority of SCD patients are of African descent; there are significant differences in antigen expression between Caucasians and African Americans (especially with regards to C, E, and K antigens). Alternatively, it has been argued that the sickle cell pathophysiology itself is responsible, due to increased inflammation and activation of innate immunity, which promotes adaptive responses upon encountering foreign antigens. To add to these existing hypotheses, Tatari-Calderone et al. have recently advanced a novel hypothesis that the immune systems of SCD patients are indeed more prone to RBC alloimmunization, due not to the biological changes as a result of SCD, but rather due to inheritance of an immunoregulatory gene that co-segregates with the sickle globin gene (Tatari-Calderone et al., 2009).

TRIM21 (Ro52;SSA1) is a known immunoregulatory gene that is in very close proximity to beta-globin, and it has recently been reported that the rs660 polymorphism in TRIM21 correlates with the age at which SCD patients become alloimmunized following RBC transfusion (Tatari-Calderone et al., 2009). A major role of TRIM21 appears to be as a negative feedback to immune activation by ubiquitinating and promoting the destruction of interferon regulatory factors (Bolland and Garcia-Sastre, 2009). The rs660 polymorphism is the result of a single base alteration in the sequence "...AGAGAT{C/T}TTTG ...". Because the rs660 polymorphism is not within the coding region of the TRIM21 gene, it has been proposed to play a role in regulating levels of TRIM21 expression (Frank et al., 1993; Tatari-Calderone et al., 2009). Thus, the overall hypothesis advanced by Tatari-Calderone et al. is that rs660C/T leads to lower levels of TRIM21 expression than rs660T/T, resulting in less negative feedback to immune activation, and higher rates of alloimmunization to RBC transfusion.

To test the hypothesis that levels of TRIM21 expression influence RBC alloimmunization, we have utilized a murine model with a well defined RBC antigenic difference between donor and recipient. Herein we report that there is no difference in frequency or magnitude of RBC alloimmunization between TRIM21 KO (Yoshimi et al., 2009) and wild-type recipients. We further report no difference in alloimmunization to a foreign MHC on transfused leukoreduced platelets (LR-PLT) between TRIM21 KO and wild-type mice. In aggregate, these studies demonstrate that TRIM21 expression is not an independent variable in the frequency or magnitude of alloimmunization to transfused RBCs or platelets in a mouse model.

2. Materials and Methods

Mice

Female C57BL6/J (B6) and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). TRIM21 knock out (TRIM21 KO; H-2^b) mice on a B6 background and HOD RBC donor mice were generated as previously described (Desmarests et al., 2009; Yoshimi et al., 2009) on a FVB background. TRIM21 KO and B6 recipients were used at 10–16 weeks (adults) or 3 weeks of age (juveniles). TRIM21 KO, HOD, and FVB mice were bred at the Emory Division of Animal Resources Animal Husbandry Service. All mice were housed in Emory University Department of Animal Resources facilities and procedures were performed according to approved IACUC protocols.

Treatment of mice to induce inflammation

A subset of mice were injected i.p. with 100 micrograms of poly (I:C) (Amersham Biosciences, Sweden) dissolved in Dulbecco's Phosphate Buffered Saline (DPBS), 4 hours prior to transfusion. Control mice received the same volume of DPBS.

Isolation and transfusion of donor RBC and platelet units

Whole blood from transgenic HOD donors was collected in a 1:8 ratio of RBC to ACD as previously described (Desmarests et al., 2009). The blood was washed three times with DPBS and centrifuged at 1,350 rpm ($394 \times g$) for 10 minutes. The packed RBCs were re-suspended in 1x DPBS at a 1:5 dilution (100 μ l packed RBCs in a total volume of 500 μ l DPBS and 60 μ l of packed RBCs in a total volume of 300 μ l DPBS were given to adults and juveniles, respectively). In some experiments, whole blood was leukocyte reduced (LR) using a RBC specific Pall Neonatal Leukoreduction filter prior to washing. Leukocyte reduced platelet units were isolated as previously described (Patel et al., 2009); briefly, platelet rich plasma was isolated by centrifugation and was passed over a Neonatal Purecell PL High Efficiency Leukocyte Reduction Filter (Pall corp.) The leukoreduced platelets were centrifuged and resuspended at a concentration of 2×10^8 platelets/ml, followed by transfusion of 10^8 platelets per recipient. All transfusions were given by tail vein.

Analysis of humoral alloimmunization

Antibody responses to transfused HOD RBCs or BALB/c LR-PLT were evaluated by indirect immunofluorescence using flow cytometry (FACS). Serum was diluted as indicated in FACS buffer and incubated with HOD RBCs or BALB/c splenocyte targets. Control targets consisting of FVB RBCs or, B6 splenocytes (SPLs) were used for HOD or BALB/c targets, respectively. All incubations were for 30 minutes at 4°C. Samples were then washed three times with 100 μ l FACS buffer, and were incubated for 30 minutes in a 1:100 dilution of allophycocyanin (APC) conjugated goat anti-mouse Igs in FACS buffer. B cells in the BALB/c or B6 SPL targets were excluded by staining with FITC anti-mouse CD19 (BD Pharmingen, 1:50 in FACS buffer). All samples were run on a FACS Caliber and analyzed

on FlowJo 8.8.2. Mean fluorescence intensity (MFI) was used to indicate the presence of antibody bound to cell targets, with adjusted MFI being defined as the mean fluorescence intensity of serum crossmatched with control RBCs or splenocytes subtracted from the signal of serum crossmatched with target RBCs or splenocytes.

Statistical Analysis

Adjusted mean fluorescence intensity was compared between groups using a two way ANOVA with a Bonferroni post-test (Graphpad Prism), with a statistically significant value being $p < 0.05$.

3. Results

Rate and magnitude of RBC alloimmunization is independent of TRIM21 expression

To test the hypothesis that TRIM21 expression is an independent variable in alloimmunization to an RBC antigen, HOD RBCs were transfused into either TRIM21 KO mice or wild-type recipients (HOD RBCs express hen egg lysozyme, a portion of ovalbumin, and human Duffy^b). Anti-HOD antibodies were assayed two weeks following transfusion by indirect immunofluorescence using HOD RBCs as targets and antigen negative wild-type FVB RBCs to control for background staining. Responders to HOD RBCs were defined as having an MFI of two standard deviations above the mean of sera cross-matched to control FVB RBCs. Combined results of three experiments demonstrated alloimmunization to HOD in 13/14 TRIM21 KO mice (93%) and 15/15 B6 recipients (100%), (Figure 1A). Although there were several outliers, overall there was no increase in the magnitude of anti-HOD alloantibody responses in recipients lacking TRIM 21 compared to wild type controls; in contrast, TRIM 21 KO recipients made lower levels of anti-HOD ($p = 0.02$).

To test the effects of TRIM21 on RBC alloimmunization in juvenile mice, 3 week old TRIM 21 KO or B6 recipients were transfused with a weight adjusted volume of HOD RBCs. Combined data from three experiments demonstrated low levels of detectable anti-HOD antibody in 8/15 TRIM21 KO mice (53%) and 10/15 B6 mice (67%). There was no significant difference in the magnitude of anti-HOD response between the two groups ($p = 0.6$) (Figure 1B). Overall, the frequency and magnitude of RBC alloimmunization in juvenile mice were substantially lower than similar data from adult-immunized mice, consistent with the blunted response of the young and/or developing immune system. Together, these data demonstrate that within the juvenile populations, the absence of TRIM21 does not alter the frequency or magnitude of alloimmunization against the HOD antigen on transfused RBCs.

Inflammation with poly (I:C) does not alter relative rates or magnitude of alloimmunization to RBCs in TRIM21KO vs. wild-type mice

We hypothesized that transfusion in the context of ongoing inflammation involving interferon regulatory pathways would be a sensitive approach to measure biological changes in alloimmunization due to decreased TRIM21. We tested this hypothesis utilizing poly (I:C), a double stranded RNA that has previously been shown to enhance RBC alloimmunization (Hendrickson et al., 2006; Hendrickson et al., 2008). and that induces the same IRF family members downregulated by TRIM 21 (Bolland and Garcia-Sastre, 2009). Adult TRIM21 KO mice or wild-type B6 mice were treated with poly (I:C) 4 hours prior to transfusion with HOD RBCs. Data from three combined experiments demonstrated that 13/15 TRIM21 KO (87%) and 10/10 B6 (100%) recipients had detectable levels of anti-HOD alloantibodies, with no difference in magnitude of alloimmunization between the groups (Figure 2, $p = 0.1$).

Alloimmunization against transfused PLTs is not altered in TRIM21 KO mice

Similar to RBC transfusions, transfusions of PLTs can result in humoral alloimmunization, most commonly against MHC I alloantigens. To test the role of TRIM21 in this process, TRIM21 KO or wild-type B6 mice were transfused with leukoreduced PLT products (LR-PLT) from BALB/c donor mice. Because BALB/c mice have an H-2^d MHC haplotype and B6 mice have a H-2^b MHC haplotype, anti-H2^d is the dominant antibody response in this setting. Mice received three transfusions, each a week apart, and the presence of anti-BALB/c alloantibodies in recipient sera was assessed by indirect immunofluorescence using BALB/c splenocytes as targets and B6 splenocytes as negative control cells to establish background staining. Responders were identified as having a MFI of two standard deviations above the mean of sera cross-matched to B6 spleen cell targets.

Data from three combined experiments demonstrated a statistically significant response rate of 7/15 (47%) and 5/15 (33%) in TRIM21 KO and B6 recipients, respectively. In addition, each group had 4 strong responders (Figure 3). This BALB/c into B6 strain combination is known to result in moderate responses to platelet antigens, as observed here, which is optimal for detecting increases or decreases. Thus, analogous to adult and juvenile recipients of HOD RBC transfusions, no statistically significant differences were observed in the frequency and magnitude of PLT alloimmunization between TRIM21 KO and B6 recipients.

4. Discussion

The described studies were undertaken in response to the novel hypothesis proposed by Tatari-Calderone et al suggesting genetic polymorphisms in the TRIM 21 gene of transfused patients with SCD influenced the generation of RBC alloantibodies, particularly in patients under the age of 5 years at the time of transfusion. Our data comparing TRIM21 KO and wild type mice do not support this hypothesis. Specifically, there was no observable difference in kinetics or magnitude of humoral alloimmunization to transfused RBCs in juvenile or adult recipients with a targeted deletion of the TRIM21 gene (Yoshimi et al., 2009). Furthermore, there was no difference observed in the enhancement of alloimmunization in response to inflammation with poly(I:C), an agent known to activate IRF family members, upon which TRIM21 exerts its immunoregulatory effects. Finally, we did not detect a difference using different antigenic stimuli (RBCs as well as platelets). Together these data reject the hypothesis that decreased TRIM 21 expression alone increases RBC or platelet alloimmunization in the described model systems.

The initial basis for Tatari-Calderone et al hypothesizing an effect of TRIM21 upon RBC alloimmunization was the physical proximity of the TRIM21 immunoregulatory gene to the beta-globin gene. Their data, suggesting an impact of a TRIM21 C/T gene polymorphism on RBC alloimmunization only in children under 5 years of age, were interpreted as suggesting a potential role for TRIM21 in affecting the rate at which the immune system matures. This polymorphism was suggested to decrease TRIM21 expression, thus resulting in less negative feedback on IRF pathways and enhanced immune maturation. If TRIM21 plays such a role in mice, we did not detect it in 3 week old recipients, which were demonstrably still in the midst of immune system development, due to decreased immune responses compared to adult mice.

The importance of our studies is that they allow a reductionist isolation of a single variable that might influence alloimmunization following RBC or platelet transfusions. The data lend support to rejection of the human data as representing a causal effect and suggest a correlation. However, there are also limitations to the current approach and numerous caveats that must be considered in the interpretation. First, one must always consider the degree to which murine biology reflects human biology. Second, one must consider that the

TRIM21KO mice utilized in these studies did not have sickle cell disease, nor were they multiply transfused. Third, it must be considered that unlike the reduced levels of TRIM21 seen in humans, these murine recipients were completely deficient in TRIM21. Finally, although it is known that the TRIM21KO strain utilized in these studies has immune dysfunction and dysregulated cytokine expression, it cannot be assumed that similar results would necessarily be seen in a different strain of TRIM21KO mice (developed in parallel by Espinosa et al) (Espinosa et al., 2009).

5. Conclusions

In summary, the data presented herein isolate TRIM21 expression as an independent variable and test the impact of its absence on alloimmunization to RBCs and PLTs in juvenile and adult recipients. Although we cannot rule out complications due to compensatory pathways in the TRIM21KO mice, the presence of dysregulated cytokines indicates that the knockout mice have an altered phenotype (Yoshimi et al., 2009) and that TRIM21 has a non-redundant role in immune regulation. In this context, we conclude that decreased expression of TRIM21 is not alone sufficient to regulate alloimmunization to RBCs or platelets in otherwise healthy animals or in animals exposed to an inflammatory stimulus prior to transfusion.

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Figure 1. Adult and juvenile TRIM21KO mice do not have increased antibody responses to alloantigen on transfused RBCs

Wild-type (B6) mice or TRIM21KO mice were transfused with donor RBCs expressing the HOD antigen. 2 weeks post-transfusion, serum was collected and anti-HOD responses were monitored using indirect immunofluorescence (1:5 dilution). Antibody binding to HOD RBC targets was measured by flow cytometry and background was subtracted using wild-type FVB control RBCs (adjusted MFI). No increased immunization was seen in either adult (1A) or juvenile (1B) TRIM21KO mice compared to wild-type B6 controls. In contrast, a trend towards decreased immunization was seen in adult TRIM21KO mice. The data are the combined results of three experiments (n=14–15 mice total/group).

Figure 2. TRIM21KO and wild-type mice have similar responses to RBC alloimmunization in the context of poly (I:C) induced inflammation

Wild-type (B6) mice or TRIM21KO mice were injected with poly (I:C), followed 4 hours later by transfusion with donor RBCs expressing the HOD antigen. 2 weeks post-transfusion, serum was collected and anti-HOD responses were monitored using indirect immunofluorescence (1:5 dilution). Antibody binding to HOD RBC targets was measured by flow cytometry and background was subtracted using wild-type FVB control RBCs (adjusted MFI). No increased immunization was seen in adult TRIM21KO mice compared to wild-type B6 controls. The data are the combined results of three experiments (n=15 TRIM21KO mice and 10 B6 wild-type mice).

Figure 3. TRIM 21 KO and wild-type mice have similar rates and magnitude of humoral alloimmunization to transfused platelets

Wild-type (B6) mice or TRIM21 KO mice were transfused with platelets from BALB/c donors. Three sequential transfusion were given (weekly) and serum was collected 2 weeks after the third transfusion. Antibody binding to BALB/c splenocyte targets was measured by flow cytometry (1:5 dilution of serum) and background was subtracted using wild-type B6 control splenocytes (adjusted MFI). No significant increase in frequency or magnitude of immunization was observed between TRIM21KO and wild-type mice. The data are the combined results of three experiments (n=15 mice/group).