Immunization to Minor Histocompatibility Antigens on Transfused RBC through Crosspriming into Recipient MHC Class I Pathways

James C. Zimring1* Gregory A. Hair 1, Seema S. Deshpande1, John T. Horan2

1Transfusion Medicine Program, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322

2Department of Pediatrics, Emory University School of Medicine, AFLAC center for pediatric blood and cancer disorders, Atlanta, GA 30322

* To whom all correspondence should be addressed

Please address correspondence to:

James C. Zimring, M.D., Ph.D., Transfusion Medicine Program, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Woodruff Memorial Building Suite 7301, 101 Woodruff Circle, Atlanta, GA 30322, USA (Telephone 404-712-2174, Fax 404-727-5764) Email jzimrin@emory.edu

Word count
Body = 1200
Abstract = 149

Running Title: Immunization to mHAs by RBC Transfusion

Nonstandard Abbreviations: Hen Egg Lysozyme (HEL), chloromethylbenzamido 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI), 3,3'-dihexadecyloxacarbocyanine perchlorate (DiO), Chicken Ovalbumin (OVA), succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate (LC-SPDP)
Abstract:

Increased rates of bone marrow transplant (BMT) graft rejection are observed in patients whose illnesses necessitate chronic transfusion prior to BMT, such as sickle cell disease, thalassemia, or aplastic anemia. Because BMTs in this setting are routinely HLA matched, any immunization responsible for increased rejection is likely against minor histocompatibility antigens (mHAs). It has been assumed that contaminating leukocytes in red blood cell (RBC) units are the main source of immunization to mHAs. However, in this report we demonstrate that antigens on donor RBC are presented in the MHC class I pathway of recipient antigen presenting cells, resulting in activation and expansion of recipient CD8+ T cells specific for donor mHAs. Since human hematopoietic progenitor cells express many of the known mHAs, this observation provides a mechanism by which chronic transfusion of even stringently leukoreduced RBC may result in sufficient mHA immunization to increase the frequency of BMT rejection.
Introduction:

Bone marrow transplantation (BMT) is now standard therapy for a variety of hematological malignancies and also represents a cure for certain non-malignant hematological disorders. To avoid the morbidity and mortality associated with stringent myeloablative therapies used to treat neoplasia, milder and less immunosuppressive conditioning regimens have been designed for treating non-malignant illnesses. However, high rates of graft rejection are observed in patients whose illnesses necessitate chronic transfusion of red blood cells (RBC) prior to BMT, such as sickle cell disease, thalassemia, or aplastic anemia. 1-3.

It has been proposed that chronic transfusion immunizes the recipient to multiple donor antigens, which contributes to subsequent rejection of bone marrow grafts. Because BMTs in the non-malignant setting are routinely HLA identical transplants, it is unlikely that immunization against MHC molecules plays a role in graft rejection. However, immunization against minor histocompatibility antigens (mHAs) is known to occur in response to chronic transfusion. 4. mHAs are polymorphic peptides from allelic variants of normal human proteins, which are displayed by MHC molecules and elicit a specific MHC-restricted T cell response. 5

It has been assumed that contaminating leukocytes in RBC units were the main source of immunization to mHAs. Accordingly, it had been anticipated that the recent implementation of leukoreduced products would decrease the incidence of matched related marrow graft rejection in multiply transfused patients. However, a recent analysis of patients undergoing transplantation for severe aplastic anemia performed by the International Bone Marrow Transplant Registry, suggests that this is not the case (John T. Horan, Unpublished data).

We hypothesized that transfusion of RBC is sufficient to immunize against mHAs. Although exogenous antigens are typically processed and presented through MHC class II
pathways, it has been reported that cell association results in a 50,000 fold greater efficiency of crosspriming into the MHC class I pathway\textsuperscript{6}. Thus, we hypothesized that donor RBC associated antigens would be efficiently crosspresented by recipient antigen presenting cells (APC) into MHC class I pathways.
Materials and Methods:

Crosslinking of OVA or HEL to the RBC surface. 2-pyridylldithiol groups were added to OVA (15mg/ml) or HEL (5mg/ml) by incubating with 0.6mg/ml succinimidyl 6-(3-[2-pyridylldithio]-propionamido)hexanoate (LC-SPDP) (Pierce Biotechnology, Rockford IL) in PBS with 1mM EDTA for 30 min followed by removal of unreacted LC-SPDP by dialysis. Stocks were stored at -80°C until use. RBC from donor mice were leukoreduced as previously described, which resulted in a 4-log₁₀ reduction in leukocytes. Free sulfhydryl groups were introduced onto leukoreduced RBC by incubating with 0.3 mg/ml of 2-iminothiolane (Traut's reagent) (Pierce, Biotechnology) for 30 min in Bicine-Saline buffer (0.02M Bicine, 0.17M NaCl, 0.01M NaOH pH 8.3). 3.8 mg/ml of LC-SPDP modified OVA or 1.3 mg/ml of LC-SPDP modified HEL were incubated with Traut’s modified RBC for 2 hours with occasional agitation, followed by washing with PBS.
Results and Discussion:

To test our hypothesis, we generated units of leukoreduced murine RBC that carry chicken ovalbumin (OVA) or hen egg lysozyme (HEL) as model antigens. Crosslinking of HEL and OVA to the RBC was confirmed by staining with rabbit anti-HEL or rabbit anti-OVA and analyzing by flow cytometry. (Figure 1A and 1B). To confirm that the crosslinked RBC maintained the ability to circulate, OVA-RBC or uncrosslinked RBC were labeled with the fluorescent dyes DiO and CM-DiI, respectively as previously described 7 and were transfused as a 1:1 mixture into recipient mice. 24 hours after transfusion, recipient mice were bled and transfused cells were visualized by flow cytometry (Figure 1C). Transfused OVA-RBC were circulating at this time and OVA was stable on the RBC surface (Figure 1D), however there were fewer modified cells than unmodified cells, indicating increased clearance of modified cells. Longer-term studies revealed that OVA-RBC have a decreased circulatory life-span compared to unmodified cells, but continue to circulate for approximately 2 weeks with stable OVA protein on the surface (data not shown).

To determine the quantity of OVA on the cell surface, RBC ghosts were prepared from OVA crosslinked RBC (OVA-RBC) and were then subjected to western blot analysis with anti-OVA. Under reducing conditions, OVA is easily detected from OVA-RBC (Figure 1E). In addition, essentially all of the observed OVA is covalently bound to the RBC by disulfide bonds, as only a very weak non-specific band is observed with the molecular weight of OVA under non-reducing conditions (figure 1F). A titration of soluble OVA demonstrates that 10 microliters of OVA-RBC ghosts contains approximately 50 nanograms of OVA (Figure 1E). Multiplying this amount by the volume of ghosts recovered from a given volume of OVA-RBC reveals that 100 microliters of packed OVA-RBC contains approximately 1 microgram of OVA protein.
The OT-I mouse is transgenic for a T cell receptor (TCR) that is specific for an OVA peptide 257-264 (SIINFEKL) presented by MHC class I H-2K\textsuperscript{b}. Thus, activation and expansion of OT-I T cells provides a readout for presentation of this OVA peptide. Splenocytes from OT-I mice were adoptively transferred into C57BL/6 mice congenic for Thy 1.1 (B6.Thy 1.1 mice). One day later, the mice received a transfusion of 100 microliters of OVA-RBC, HEL-RBC, soluble OVA, or no transfusion. 5 animals were included in each group that received transfusions. B10.BR (H-2\textsuperscript{k}) mice were used as RBC donors. Since B10.BR mice do not express H-2K\textsuperscript{b}, this experimental design precludes the possibility that any observed expansion of the OT-I T cells was due to direct presentation of OVA by donor cells.

Three days after the transfusion, splenocytes were isolated from the recipient animals and OT-I T cells were visualized by staining with anti-CD8 and a tetramer specific for the OT-I TCR (SIINFEKL-Tetramer) (Figure 2A-E). In mice that received OVA-RBC, OT-I T cells expanded to a total number of 4.4 million cells, which represents a 49 fold expansion of OT-I T cells compared to mice that received no transfusion (p value = 0.0002). In contrast, HEL-RBC resulted in a small expansion (2 fold (p =0.04) and no significant expansion was seen for soluble OVA (1.3 fold (p=0.07)). The observed expansion is not dependent upon the antigen being linked by disulfide bonds, as the same experiment using an irreversible maleimide-based crosslinker (Sulfosuccinimidyl 4-[p-maleimidophenyl]butyrate) gave similar results (data not shown). Because TCRs can be down modulated upon activation, staining with anti-CD8 and anti-Thy 1.2 was also used as a separate measure of OT-I T cells. Staining with anti-Thy 1.2 demonstrated the same trend, but with a slightly greater increase of OT-I T cells in the OVA-RBC transfused mice.
Together, the data presented herein indicate that RBC-associated proteins undergo crosspresentation by recipient APC into MHC class I pathways and that this results in the functional activation and expansion of mHA specific CD8^+ T cells. Human hematopoietic progenitor cells express many of the known mHAs. Thus, any protein expressed by RBC that is likewise expressed in hematopoietic cells can serve as a mHA for which an immune response may be primed by RBC transfusion and lead to increased rejection of BMT. The observation that donor RBC antigens can be presented by MHC class I of host APC and can stimulate host CD8^+ T cells provides a mechanism by which chronic transfusion of even stringently leukoreduced RBC may result in sufficient immunization to mHA transplantation antigens to cause an increased frequency of BMT rejection.
Figure Legends

**Figure 1 Chemical Cross-linking of OVA and HEL to RBC.** OVA or HEL was crosslinked to leukoreduced RBC as described (see materials and methods). HEL-RBC and OVA-RBC were stained with rabbit anti-HEL or rabbit anti-OVA, respectively (A+B). Cells were washed and incubated with goat-anti-rabbit immunoglobulin conjugated to allophycocyanin. Staining of cells was analyzed by flow cytometry. OVA-RBC were labeled with DiO and unmodified RBC were labeled with CM-DiI, as previously described 7. A mixture of labeled cells was transfused into C57BL/6 mice. 24 hours post transfusion, peripheral blood was obtained and the percentages of circulating RBC was determined (C). The same blood specimen analyzed in panel C was stained with anti-OVA as above, and following appropriate gating, fluorescence was compared on transfused OVA-RBC (solid line) vs. transfused unmodified RBC (dashed line) (D). RBC ghosts were prepared by hypotonic lysis and were subjected to western blot with anti-OVA under reducing (E) or non-reducing conditions (F). Standard curves of OVA were also generated using western blots. To control for any changes in OVA immunoreactivity as a result of the crosslinker, the OVA standard curve was generated using unreacted LC-SPDP modified OVA.

**Figure 2 Expansion of OT-I T cells in Response to Transfusion of OVA-RBC.** 20 x 10⁶ OT-I splenocytes were adoptively transferred into B6.Thy1.1 mice. One day later, mice were transfused with 100 µL OVA-RBC, 100 µL HEL-RBC or soluble OVA. One microgram of soluble OVA was used, as this was the amount of OVA contained on 100 µL of OVA-RBC (see figure 1). To control for any changes in OVA immunoreactivity as a result of the crosslinker, the
soluble OVA used was LC-SPDP modified OVA. There were 5 animals in each experimental group, with 2 animals in the control group that received no transfusion. Three days after transfusion, splenocytes were isolated from recipient animals and OT-I T cells were visualized by staining with anti-CD8 and tetramer (Panels A-E) or anti-CD8 and anti-Thy 1.2 (Panels F-J). The legitimacy of the gates used to identify OT-I cells was confirmed by staining splenocytes from mice that had received no OT-I T cells (Panels E and J). Total numbers of OT-I T cells were calculated by counting the number of splenocytes recovered from each animal and multiplying by the resulting percentages of CD8+ tetramer+ cells. Representative flow cytometry plots from individual animals are presented (Figure 2 A-J). 5 animals were included in each group that received a transfusion and the numerical averages for each group is presented in the accompanying table. Standard deviations were calculated by combining the results of all animals in a given group using total numbers of cells. P values represent two-tailed p values generated using an unpaired t test. This particular experiment has been performed 4 times with similar results.
Acknowledgements: We would like to thank Traci Chadwick for outstanding technical assistance.
References


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mercaptobutyrimidate as a cleavable cross-linking reagent and its application to the Escherichia

Figure 1
Figure 2
Immunization to minor histocompatibility antigens on transfused RBC through crosspriming into recipient MHC class I pathways

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