Loss of Red Cell Chemokine Scavenging Promotes Transfusion Related Lung Inflammation

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Non Standard Abbreviations: ALI (acute lung injury), ARDS (acute respiratory distress syndrome) AS-1 (additive solution-1), AS-5 (additive solution-5), BAL (bronchoalveolar lavage), CPDA (citrate phosphate dextrose adenine), EU (Endotoxin Unit), HEX (Hextend®), ICU (Intensive Care Unit), MIP-2 (macrophage inflammatory protein), MPO (myeloperoxidase), RBC (Packed Red Blood Cell), TRALI (transfusion related acute lung injury)
Abstract

Red cell transfusions are associated with the development of acute lung injury in the critically ill. Recent evidence suggests that storage induced alterations of the red cell collectively termed the “storage lesion” may be linked with adverse biological consequences. Using a two event model of systemic endotoxemia followed by a secondary challenge of red cell transfusion, we investigated whether purified red cell concentrates from syngeneic C57Bl/6 mice altered inflammatory responses in murine lungs. Transfusion of packed red cells (RBC) stored for 10 days increased neutrophil counts, MIP-2 and KC concentrations in the airspaces, and lung microvascular permeability when compared with transfusion of < 1 d old RBC. Because red cells have been shown to scavenge inflammatory chemokines through the blood group Duffy antigen (Fy), we investigated the expression and function of Fy during storage. In banked human RBC, both Fy expression and chemokine scavenging function were reduced with increasing duration of storage. Furthermore, transfusion of Fy⁻/⁻ RBC into Fy⁺/+ endotoxemic mice increased airspace neutrophils, inflammatory cytokine concentrations, and lung microvascular permeability compared to transfusion of Fy⁺/+ RBC. Thus, reduction in erythrocyte chemokine scavenging is one functional consequence of the storage lesion by which RBC transfusion can augment existing lung inflammation.
Introduction

Although red cell transfusions have been a mainstay in the treatment of critically ill patients, recent studies have shown an association between packed red blood cell (RBC) transfusion and increased morbidity and mortality in this cohort.\textsuperscript{1-4} Supported by the initial observations of Fowler et al., recent epidemiological data collectively suggest that red cell transfusion is a predisposing condition for the development of lung injury in the critically ill population at risk for ARDS.\textsuperscript{5} Indeed, in a cohort of critically ill patients with Acute Lung Injury (ALI), the risk of mortality increased with the number of RBC units transfused, suggesting that each transfusion serves as another “hit” or insult perpetuating lung inflammation and injury.\textsuperscript{6} Furthermore, RBC transfusion was found to be an independent predictor for the development of and mortality from ARDS in a prospective study of patients at risk for ARDS,\textsuperscript{6} and a predictor of multiple organ failure (MOF) in a cohort of trauma patients.\textsuperscript{7} Although the present epidemiological and observational studies highlight an important clinical association between red cell transfusion and ALI, the mechanisms underlying this association have yet to be fully elucidated.

Multiple factors may contribute to the development of lung injury after transfusion in at risk patients. Allogenic leukocytes of red cell transfusates have long been implicated as a source of immunomodulatory effects.\textsuperscript{8,9} However clinical trials of leukoreduction have shown conflicting results.\textsuperscript{8-10} Recent data from patients given packed red cells (RBC) during cardiac surgery shows that the risk of mortality rises with increasing age of red cells being transfused.\textsuperscript{11} This lends credence to the concept that biochemical and
morphological alterations the red cell undergoes with storage, i.e. the “storage lesion”, contributes to morbidity associated with RBC transfusion.

Independent of storage, prior studies illustrate that erythrocytes have the potential to induce biological activity through various mechanisms. For example, erythrocytes form advanced glycation end products over time, convert eicosinoid mediators, generate reactive oxygen species, and possess chemokine binding activity that can modulate an existing inflammatory state.\textsuperscript{12-17} However, the role of red cell transfusates in the perpetuation or amplification of existing inflammatory responses during critical illness remains unknown.

We have previously shown that erythrocyte Duffy antigen can scavenge chemokines in whole blood stimulated with endotoxin.\textsuperscript{18} First recognized as the receptor for the parasite \textit{Plasmodium vivax}, the Duffy antigen is a minor blood group antigen that binds multiple inflammatory CXC and CC chemokines with high affinity.\textsuperscript{12,14,19,20} Intracellular calcium flux does not occur upon chemokine binding to erythrocyte Duffy antigen and chemokines bound to erythrocytes are not accessible to circulating neutrophils.\textsuperscript{12} During inflammatory states, we have also shown that erythrocyte Duffy antigen has a more prominent role than endothelial Duffy antigen in chemokine clearance from the lung microvasculature to the systemic circulation.\textsuperscript{18} Prior observations of others suggest that red cell Duffy antigen expression may be reduced under standard blood bank conditions.\textsuperscript{21} We hypothesized that storage related alterations in Duffy antigen is one
mechanism by which red cell transfusion can have adverse biological consequences by modulating existing inflammatory responses in the lung microvasculature.
Materials and Methods

Human study subjects

Healthy volunteers between the ages of 18 and 65 years gave written informed consent for the studies in accordance with the Declaration of Helsinki. All experimental procedures involving human subjects or use of human packed red blood cells (PRBCs) have been approved by the University of Pittsburgh Institutional Review Board.

Isolation of human erythrocytes

Whole blood was obtained via venipuncture and Citrate-Phosphate-Dextrose-Adenine (CPDA, Sigma-Aldrich, St. Louis, MO, USA) solution was added to the whole blood (1 ml CPDA: 9 mls whole blood). Erythrocytes were purified using modifications of a technique described by Beutler et al.22 The whole blood was passed over 2 Sephadex G25: Microcellulose (1:3, Sigma-Aldrich, St. Louis, MO, USA) columns in tandem. The columns were washed with 10x the volume of sterile PBS (Baxter Healthcare Corporation, Deerfield, IL, USA). The eluent was centrifuged at 1000 g x 4 minutes at 4°C. The erythrocyte pellet was washed 3 times with cool sterile PBS. The RBC concentrates were stored at 4°C in 50 ml polypropylene tubes (BD Biosciences, San Jose, CA, USA).

Surface expression of Erythrocyte Duffy Antigen

Packed Red Blood Cells (RBCs) stored in additive solution (AS)-5 were obtained from the Institute for Transfusion Medicine (Pittsburgh, PA) and stored for various time periods (D 1-54). Human erythrocytes from healthy volunteers were purified and stored.
in AS-1 at 4 °C for 11 and 31 days. 1x 10^6 erythrocytes were suspended in FACS buffer (2% Fetal Bovine Serum, PBS) and incubated with 1 ug anti-Fy6 mouse monoclonal antibody (BD Biosciences-Pharmingen, San Jose, CA, USA) or IgG1 isotype control (R&D Systems, Minneapolis, MN, USA) for 30 minutes on ice in the dark. The cells were washed 3x and resuspended in FACS buffer. The erythrocytes were subsequently labeled with 1 ug FITC conjugated goat anti-mouse F(ab')2 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 30 minutes. The cells were again washed three times. FACS analysis was performed (FACSCalibur, BD Biosciences, San Jose, CA, USA). Data was analyzed using FCS Express Software (De Novo Software, Los Angeles, CA, USA).

Experimental Animals

C57BL/6J animals were purchased from The Jackson Laboratory. The generation and breeding of Duffy KO mice has been previously described. All experimental procedures were performed in 8-12 week old male mice. Animal studies were conducted in accordance with the Institutional Animal Care and Use Committee at the University of Pittsburgh. The animals were housed and maintained in a pathogen free environment by the Department of Laboratory and Animal Research at the University of Pittsburgh.

Isolation of murine erythrocytes

C57BL/6J or KO mice were euthanized with intraperitoneal pentobarbital (120mg/kg). Whole blood was drawn via intracardiac puncture and collected into sterile 1-ml tuberculin syringes. CPDA (Sigma-Aldrich, St. Louis, MO, USA) solution was
immediately added to the whole blood (1:9). The whole blood was passed over 2 Sephadex G25: Microcellulose (1:3, Sigma-Aldrich, St. Louis, MO, USA) columns in tandem. The columns were washed with 10x the volume of sterile PBS (Baxter Healthcare Corporation, Deerfield, IL, USA). The eluent was centrifuged at 1000 g x 4 minutes at 4°C. The erythrocyte pellet was washed 3 times with cool sterile PBS and concentrated to a hematocrit of 60-70% and stored in 50 ml polypropylene tubes (BD Biosciences, San Jose, CA, USA) at 4°C until transfusion. Viability was assessed prior to transfusion using Trypan blue. For in vivo studies using < 1 d versus 10 d old stored blood, erythrocytes were purified as detailed above, and stored in AS-5 (Terumo Corp, Somerset, NJ, USA) at 4°C at a ratio of 3 parts RBC to 1 part AS-5.

Erythrocyte Chemokine Binding Assays

Erythrocyte chemokine binding was assayed using modifications of the techniques of Darbonne et al. For studies involving human erythrocytes stored under blood bank conditions, erythrocyte samples were obtained from PRBC units containing AS-5 (Institute for Transfusion Medicine, Pittsburgh, PA, USA). Six units of PRBC were sampled on days 12 and 27 of storage. Just prior to day of experiment, RBC were isolated and purified through sephadex-microcellulose columns as previously described. Chemokine binding studies were performed within 24 hours of RBC purification. For studies using RBC from human volunteers, RBC were purified as described above and stored for 1 or 13 days.
Erythrocytes were counted manually and then incubated for 30 minutes at room
temperature with chemokine binding buffer (DMEM + 0.1% HSA) in the presence or
absence of 2 nM murine or human recombinant CCL2, murine MIP-2, human CXCL8 or
CXCL1 (PeproTech, Rocky Hill, NJ, USA). The erythrocyte + chemokine suspension
was layered upon a 30% sucrose cushion and centrifuged for 3 minutes at 13000 g. The
supernatant was collected and frozen at -80 ºC. The erythrocyte pellet was lysed using
red cell lysis buffer (500mM NaCl, 0.1% Triton-X 100 and 0.25% BSA) and then frozen
at -80 ºC. Chemokines contained in the supernatant or cell lysates were measured by
ELISA (R&D Systems, Minneapolis, MN, USA).

Animal preparation and experimental protocol

Tail vein injection of LPS

Mice were injected with 1.5 mg/kg LPS (from E. coli 011:B4, List Biological
Laboratories, Campbell, CA, USA) via tail vein.

Transfusion of erythrocytes

Murine packed red cells were passively warmed to room temperature prior to transfusion.
C57BL/6J mice were transfused 8ul/g erythrocytes, sterile PBS or sterile Hextend® via
tail vein. For studies involving both LPS and transfusate administration, mice received 8
ul/g erythrocytes from C57BL/6J mice (< 1d RBC, 1-2 d RBC, or 10d old RBC), KO
mice (1-2d RBC) or 8 ul/g sterile Hextend® 2 hours following systemic LPS
administration. Where specified, < 1 d RBC and 10 d old RBC were washed twice with
20x the volume of cool sterile PBS and concentrated to a Hct of 60% immediately prior to transfusion.

**Mouse necropsies, BAL, Cell Counts, and Total Protein Measurements.**

Animals were sacrificed at specified time points with 120 mg/kg intraperitoneal pentobarbital. Mouse necropsy, lung tissue processing and BAL have been previously described in detail.\(^{18,24}\) Cell counts and differentials were determined by counting 200 consecutive cells from cytospin preparations stained with Diff Quik. BAL total protein measurements were performed using the Bradford Assay (Thermo Fisher Scientific Inc., Rockford, IL, USA).

**Lung Histology.** The whole lung was inflated with 3 mL of 4% paraformaldehyde, and paraffin embedded. H&E staining was performed on sections cut at 4-6 micron thickness. Images were acquired using Axiocam software (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). Immunofluorescence was performed using rat anti-mouse GR-1 antibody (Cedarlane Laboratories, Ltd., Burlington, NC, USA), rat IgG as control (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and goat anti-rat Alexa 488 conjugated secondary antibody. Images were acquired using an Olympus Provus Ax70 microscope.

**Lung Myeloperoxidase determination.** Whole lung myeloperoxidase (MPO) activity was determined using the method described by Goldblum et al\(^ {25}\). The left lung was homogenized in 50 mM potassium phosphate buffer (pH 6.0) with 0.5%
hexadecyltrimethylammonium bromide (HTAB, Sigma-Aldrich, St. Louis, MO, USA) on ice. The homogenate was centrifuged at 3000 g for 10 minutes. The pellet was re-homogenized, sonicated and then freeze thawed (3 times). The suspension was centrifuged at 9,500 g for 20 minutes. The supernatant was assayed with 50mM potassium phosphate buffer containing 16.7 mg/ml o-dianisidinedihydrochloride (Sigma-Aldrich, St. Louis, MO, USA) and hydrogen peroxide. Absorbance at 460 nm at 10 minutes was measured using a spectrophotometer.

*Plasma and BAL cytokine determination.* Plasma and BAL cytokine concentrations were measured using a Multiplex Kit (R&D Systems, Minneapolis, MN, USA).

*Statistics.* Results are reported as the mean ± standard error of the mean. 2 tailed Student’s t test, Mann-Whitney Rank Sum, ANOVA or Spearman’s rank order correlation was used where appropriate to determine significance. Statistics were performed using SigmaPlot 10 software (Systat Software Inc., Chicago, IL, USA). P values less than or equal to 0.05 were determined to be significant.
Results:

Transfusion of red cell concentrates does not alter lung inflammation in unstimulated mice.

We developed a method of purification of erythrocytes using modifications of a technique described by Beutler et al that removes platelets and leukocytes. This method resulted in > 99% pure erythrocytes, and did not yield significant LPS contamination in packed red blood cells (Supplemental Data, Figure 1). We examined pH, K+, lactate, 2,3-DPG, and free hemoglobin (Hgb) levels in stored murine erythrocytes. The pH and free Hgb levels in stored murine purified RBC were comparable to levels in banked human blood at later time points (Supplemental Data, Table 1). The 2,3-DPG levels in murine blood were higher than in human blood.

C57BL/6J mice wildtype for the Duffy antigen and naive to any prior stimulus were transfused 8ul/gram of purified syngeneic erythrocytes stored for 1-2 days. This volume/weight of blood approximated 2 units of RBCs (560 ml/70 kg person). To control for the colloid effect of volume infused, mice were also transfused with sterile 6% Hetastarch in Lactated Electrolyte Injection (Hextend®), an infusate used for rapid volume expansion in the critical care setting. Transfusion of 1-2 d RBC did not increase bronchoalveolar lavage (BAL) cytokines, airspace neutrophil counts, or total neutrophil content in the lungs (as measured by MPO activity) of unstimulated mice 4 h following transfusion, when compared with PBS or Hextend® (HEX) (Figure1A-B). Based upon these observations, we concluded that our method of RBC purification did not elicit inflammatory responses in vivo. Hextend® infusion elicited similar responses in lung
cytokines, airspace neutrophil counts, and MPO activity as sterile phosphate buffered saline (PBS) transfusion (Figure 1A-B). Therefore, Hextend® was chosen as the control infusate for subsequent experiments.

Systemic LPS increases lung cytokine responses and neutrophil entrapment but does not promote accumulation of neutrophils in the airspaces

LPS administration by tail vein produced a systemic inflammatory response marked by hypothermia and elevated systemic cytokines (Supplemental Data, Figure 2A-B). Local activation of cytokines occurred in the lungs, as marked by increases in BAL MIP-2, KC, IL-6, and TNF-α (p<0.05, Figure 1C). However, mice showed no neutrophils in the airspaces 4h following onset of endotoxemia, as determined by BAL polymorphonuclear (PMN) cell counts (Figure 1D). This was despite increases in lung MPO activity, a marker of total lung neutrophil content, from baseline levels. Thus, systemic LPS increased lung cytokine responses and neutrophil entrapment in the microvasculature, but an additional stimulus is required for neutrophil migration into the airspaces to occur.

Erythrocyte transfusion increases BAL chemokine MIP-2 and airspace neutrophil recruitment during endotoxemia.

We next determined whether red cell transfusion can modulate existing inflammatory responses. We developed a 2 event model whereby red cell transfusion was administered following the onset of systemic endotoxemia. We transfused mice 2 h following systemic LPS challenge with either colloid control infusion or purified RBC stored for 1-2 d. While plasma concentrations of MIP-2 showed an early increase at 2 hours (994 v 551

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pg/mL, p=0.044, Supplemental Data, Figure 3A) and KC showed a delayed increase at 16 h following RBC transfusion when compared with Hextend® control (20741 v 9639 pg/mL, p=0.035, Supplemental Data, Figure 3B), there were no significant differences in plasma chemokine concentrations 4 hours after transfusion. In the lung compartment, however, BAL MIP-2 was higher in the RBC group when compared with Hextend controls 4 h after transfusion (50 v 15 pg/mL, p=0.009; Figure 2A). The increase in BAL MIP-2 concentrations was associated with a 9 fold increase in airspace neutrophils in the 1-2 d RBC group when compared with Hextend® controls (2433 v 255 pg/mL, p<0.001; Figure 2B). In contrast, total neutrophil content in the lungs, as measured by MPO activity in homogenates, was not measurably different between the two groups, Figure 2B. Lung microvascular permeability, as measured by BAL total protein, was higher in the RBC transfused group but this difference was not significant (411 v 358 ug/mL p=0.122, data not shown). Immunofluorescence staining with anti-Gr1 of the lungs 4 hours following RBC transfusion confirmed increased numbers of neutrophils located within the vessels, interstitium, and airspaces when compared with the Hextend® transfused group (Figure 2 C-D). Thus, transfusion of 1-2 d stored RBC increased airspace concentrations of the Duffy ligand MIP-2 and was associated with detectable increases in neutrophil counts during endotoxemia.

Effect of erythrocyte storage duration on airspace PMN and lung microvascular permeability.

We determined whether increased duration of RBC storage was associated with increased airspace neutrophil migration and lung microvascular permeability. It has previously
been shown that rat erythrocytes stored for 1 week show storage related alterations similar to human erythrocytes stored for 4 weeks, suggesting that the red cell storage lesion occurs more rapidly in rodent erythrocytes than human erythrocytes. Given these concerns and our own observations of accelerated lysis of murine RBC beyond 14 days, we chose to transfuse murine RBCs stored for 10 days. Mice transfused with 10 d RBC showed increased airspace neutrophil counts, when compared with mice transfused with RBC stored for less than 1 d (2748 v 236, p=0.015, Figure 3A). This increase in airspace neutrophil counts was associated with increased lung microvascular permeability (Figure 3B). Washing the purified RBC immediately prior to transfusion did not abrogate either the increases in airspace neutrophil counts or lung microvascular permeability (Figure 4A-B), suggesting that the effect is related to the property of the red cell itself. Furthermore, transfusion of washed 10 d RBC during endotoxemia increased BAL concentrations of the CXC chemokines MIP-2 and KC (927 pg/mL versus 227 pg/mL, p=0.021 for MIP-2; 4371 pg/mL versus 2735 pg/mL, p<0.001 for KC; Figure 4C). Collectively, these findings indicate that storage dependent alterations of the erythrocyte can promote lung injury during endotoxemia.

*Transfusion of Duffy KO erythrocytes amplifies lung inflammation in endotoxemic mice.*

We have previously shown that the erythrocyte membrane protein Duffy antigen scavenges chemokines from the lung microvasculature during inflammatory states. Elevated BAL concentrations of the CXC chemokines MIP-2 and KC in mice transfused with 10 d old RBC suggested that stored erythrocytes do not scavenge chemokines as well as native erythrocytes.
We determined whether complete loss of chemokine scavenging on red cell transfusates (i.e. transfusion of Duffy KO erythrocytes) could further augment lung inflammatory responses. Endotoxemic mice were transfused with either Duffy KO or WT red cell concentrates stored for 1-2 d and studied 4 hours following transfusion. In the lung compartment, transfusion with KO RBC significantly increased BAL MIP-2, KC, IL-6 and TNF-α (p<0.05 for all comparisons, Figure 5A) and airspace neutrophil counts (22864 versus 1434, p<0.001, Figure 5B) when compared with animals transfused with Duffy WT red cells. This increase in airspace neutrophil migration was also associated with an increase in lung microvascular permeability (p=0.034, Figure 5C). Thus, during endotoxemia, the complete loss of Duffy chemokine scavenging on RBC transfusates further augmented existing lung inflammatory responses and increased microvascular permeability.

_red cell chemokine scavenging is reduced with storage._

To determine whether red cell chemokine scavenging is reduced with storage, we measured the ability of red cells of varying storage duration to scavenge soluble chemokine in vitro. Murine erythrocytes incubated with 2 nM recombinant CCL2 showed a time-dependent loss of chemokine scavenging function during storage (i.e. higher soluble CCL2 concentrations) (Figure 6A). To confirm alterations in soluble chemokine concentrations was Duffy dependent, we incubated 2 nM recombinant CCL2 with Duffy KO or wildtype (WT) erythrocytes stored for either 11, 17, or 21 days. We did not examine surface expression of Duffy antigen on stored murine erythrocytes due to
lack of a reliable antibody recognizing murine Duffy antigen. Duffy WT red cells showed reduced chemokine scavenging (i.e. higher soluble CCL2 concentrations) with increased storage time; soluble chemokine concentrations reached levels closer to those of Duffy KO red cells (p = 0.006, Figure 6B). Consistent with these findings, there was a reduction in membrane associated chemokine as erythrocyte lysate chemokine concentrations decreased with storage time (p=0.011, Figure 6B). Because Duffy is a promiscuous chemokine binding protein, we also tested red cell chemokine scavenging of MIP-2, a CXC neutrophilic chemokine. We noted similar reductions in chemokine scavenging (i.e. higher soluble MIP-2 concentrations) by WT erythrocytes over time, with MIP-2 reaching levels closer to those of Duffy KO red cells (Figure 6C).

*Human erythrocyte chemokine scavenging and Duffy antigen expression are reduced with storage.*

We determined whether the observed storage dependent alterations in murine red cell chemokine scavenging occurred in human red cells. We measured the ability of purified human red blood cells stored for various time points to scavenge soluble chemokine (Figure 7A). We observed an increase in soluble CCL2 concentrations with increased erythrocyte storage time (Figure 7A). This was associated with reduced membrane bound chemokine, as erythrocyte cell lysates showed lower CCL2 concentrations with increased storage time (Figure 7A). We next tested the ability of packed red blood cells (PRBCs) obtained from the blood bank to scavenge CXC chemokines (Figure 7B). Erythrocyte chemokine scavenging of CXCL1 was significantly reduced (p=0.008) where as scavenging of CXCL8 was unaltered (Figure 7B).
Several plausible reasons exist as to why alterations in red cell CXCL8 scavenging were not observed despite reductions in CCL2 and CXCL1 scavenging. CXCL8 may bind Duffy antigen with lower affinity than CCL2 or CXCL1. In cross-competition binding studies performed using a human endothelial cell line stably expressing the Duffy antigen, we have previously shown that the ability of CXCL1 and CCL2 to compete off radiolabeled CXCL1 from Duffy binding sites is greater than by CXCL8 (cold competitor Kd 5-7 nM versus 36 nM). Therefore, alterations in CXCL8 scavenging with storage duration may be difficult to observe without performing the study across multiple concentrations of ligand. Furthermore, reductions in red cell CXCL8 scavenging may occur either before or after the time period studied, and the comparisons made between D12 and D27 banked red cells did not capture the difference. It is also possible that storage does not substantially reduce CXCL8 scavenging but it significantly impairs red cell binding to other neutrophilic chemokines such as CXCL1 as well as CC chemokines such as CCL2. This alternative scenario could occur if storage induces changes in the tertiary structure of the Duffy antigen (ie. oxidative modification, non-enzymatic glycosylation) that differentially affects the chemokine binding pockets for CXCL1 and CXCL8. Further studies to determine alterations in erythrocyte chemokine scavenging would necessitate formal ligand binding studies to calculate the Kd and number of receptor binding sites with duration of storage for the different chemokines mentioned.

To assess whether the observed reduction in Duffy function was associated with alterations in surface expression, we examined Fy expression on purified red cells from
volunteers or from red cell units using Fy6 monoclonal antibody (Figure 7C-D). There was detectable reduction in Fy antigen expression on red cells from different volunteers with increasing duration of storage (Figure 7C). Loss of Fy expression on banked red cells from unit bags correlated with increased duration of storage ($r= -0.762$, $p=0.021$, Figure 7D), although absolute reduction was modest.
Discussion

Several major conclusions can be drawn from the findings presented in this study. In the presence of systemic and local inflammatory states, transfusion of purified stored syngeneic red cells can elicit a modest increase in lung MIP-2 concentrations and provide a secondary signal for entrapped neutrophils in the lung microvasculature to migrate into the airspaces. With increased duration of RBC storage, there is increase in BAL CXC chemokines, airspace neutrophil accumulation, and lung microvascular permeability. This response produced in the lungs following RBC transfusion appears to be related to the property of the red cell itself, as washing the RBC immediately prior to transfusion did not abrogate the response. Duffy antigen, a red cell transmembrane protein, showed impaired chemokine scavenging with increased duration of RBC storage. Furthermore, the complete loss of chemokine scavenging in RBC transfusates augmented existing lung inflammatory responses and promoted injury in recipients. These findings implicate the red cell “storage lesion” as a modulator of lung inflammatory responses and impaired erythrocyte chemokine scavenging as one functional consequence of this lesion.

Multiple aspects distinguish our model from prior studies of transfusion related acute lung injury (TRALI). In a previous model, both the plasma and lipid fraction from stored platelets and RBC induced injury in perfused rat lungs ex vivo. In another model, TRALI was induced by MHC Class I antibodies in vivo. Our model is the first in vivo model to examine the unique properties of stored erythrocytes in modulating existing inflammatory states. Although our method of red cell storage and processing does not exactly replicate clinical RBC preservation conditions, our goal was to examine the properties of the red cell itself isolated from other constituents of red cell concentrates.
during storage and its effects in vivo following transfusion. There may be absolute time differences in duration of storage and degree of functional reduction in red cells between mice and humans, but our findings in banked human red cell units showing reduction in chemokine scavenging with increasing duration of storage attest to the relevance of our murine model. The recent epidemiological studies associating red cell transfusion with increased morbidity and mortality and controversies regarding the use of stored blood in critically ill patients highlight the need for a clinically relevant model of packed red cell transfusion during critical illness.\textsuperscript{11,30} Because this is not a model of severe acute lung injury or a lethal one, our model may be a more relevant representation of what occurs in at risk populations in the critical care setting (ie. the majority of these patients are not diagnosed with transfusion related acute lung injury (TRALI) by the current definition).

Packed red blood cells undergo a series of changes collectively known as the “storage lesion”. Included in the “storage lesion” are alterations of the red cell membrane such as loss of discoid shape, vesicle formation, membrane protein oxidation and lipid peroxidation and biochemical modifications such as loss of 2, 3-DPG.\textsuperscript{21,31-38} It has also been previously suggested that surface expression of erythrocyte Duffy antigen is decreased with red cell storage and Duffy antigen has been identified on exocytic vesicles from stored erythrocytes.\textsuperscript{39-42} Consistent with our findings, others have shown that banked human RBCs undergo loss of Duffy expression and chemokine scavenging function.\textsuperscript{21}
In banked human red cell units, we found that the overall reduction in surface Duffy expression is modest and may not fully explain the significant reductions in chemokine scavenging of CXCL1 and CCL2. One possible explanation is that Duffy antigen undergoes modifications during storage which alter regions critical for chemokine binding function but that the linear epitope recognized by the Fy6 monoclonal antibody remains relatively preserved. This could also explain why scavenging of CXCL1 and CCL2 were significantly reduced but not CXCL8, as it would be expected that the chemokine binding “pocket” created by the tertiary structure is different for different ligands.

In the murine studies, the complete loss of erythrocyte chemokine scavenging function during endotoxemia results in significantly higher local chemokine concentrations. As red cells traverse the lung microvasculature, the inability of a red cell to scavenge chemokines rapidly creates a local chemotactic gradient that favors entrapped neutrophils in the lung microvasculature to migrate into the airspaces. This is supported by significantly higher BAL chemokine concentrations in mice transfused Duffy KO erythrocytes and prior findings of Darbonne and colleagues that showed the ability of red cells to deplete chemokine across the compartments of a diffusion chamber. 12

Loss of chemokine scavenging on stored red cells may also account for the observed lung injury following transfusion of stored 10 d washed RBC. This is supported by the in vitro findings of impaired chemokine scavenging with increasing red cell storage time and the
in vivo findings of elevated BAL chemokines in mice transfused 10 d RBC when compared with < 1 d RBC.

Alternatively, lung injury after red cell transfusion in endotoxemic animals may be secondary to a Duffy independent mechanism. It is possible that biologic response modifiers other than Fy present in the soluble fraction of red cell concentrates mediate the lung inflammatory response, as washing of RBC immediately prior to transfusion may not have removed all soluble mediators present.43,44 Other storage induced alterations of the red cell may provoke an inflammatory response such as advanced glycation endproducts (AGE), which have been implicated in several disease states, and previously identified in stored RBC.45 Free heme and hemoglobin may further incite inflammatory states in RBC transfused animals. Heme has been shown to promote inflammatory responses in vitro and neutrophil migration in an in vivo model of peritonitis.46,47 Free hemoglobin released during hemolysis has been shown to induce endothelial cell dysfunction and vasculopathy.48-50 While we measured plasma free hemoglobin to assess the degree of hemolysis in transfused animals, we did not see significant differences in the groups examined at the 4 h time point (data not shown). However, hemolysis by-products in transfusates require further study, given its many biological effects on the vascular endothelium and neutrophils.

Still another possible explanation is that the lung injury following red cell transfusion is secondary to mechanical injury and vascular occlusion. Reduced erythrocyte deformation, amplified echinocyte formation and increased aggregability of transfused
erythrocytes could lead to injury to the pulmonary vasculature. Furthermore, loss of S-nitrosohemoglobin in stored erythrocytes and an inability of transfused erythrocytes to vasodilate the local vasculature adequately may lead to increased ischemia, obstruction and occlusion in the pulmonary microcirculation.\textsuperscript{51,52} These mechanisms are not mutually exclusive as transfusion of stored red cells during endotoxemia may both directly incite injury (through multiple mechanisms), and sustain lung injury through an inability to clear excess chemokines.

In summary, we provide evidence that transfusion of stored erythrocytes amplify existing lung inflammation. We also demonstrate that increased duration of RBC storage can promote lung injury and implicate the loss of Duffy antigen as one functional consequence of the red cell “storage lesion”. Red cell transfusion remains a modifiable risk factor for the development of ARDS in the critically ill. A greater understanding of the storage lesion as it relates to in vivo consequences is needed so that strategies to conserve red cell integrity during storage can be optimally addressed.
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Authorship

Contribution: N.M. performed the research, analyzed the data, and wrote the paper. Z.X., T.O., M.F. performed research and analyzed the data. M.H., M.R., X.H.L, and M.R. performed aspects of the research. D.T. provided vital reagents and interpreted data. A.C. analyzed and interpreted data. J.S.L conceived and designed the studies, analyzed and interpreted data, and wrote the paper.

Conflict of interest disclosure: All authors have no conflict of interest to declare.

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References


Figure Legends

Figure 1. Lung inflammatory responses following RBC transfusion or systemic LPS in naive mice. (A) BAL cytokine concentrations in PBS, Hextend®, and RBC transfused groups in unstimulated mice (P= 1 for all cytokines measured). (B) BAL total mononuclear cell counts (P=0.930), total neutrophil counts (P=0.815) or lung MPO activity (P=0.294). (C) BAL cytokines MIP-2, KC, and IL-6 were elevated from baseline at 2 and 4 hours after LPS injection (P= 0.024 for MIP-2, KC, and IL-6 at 2 hours, P= 0.012 for MIP-2, P = 0.002 for KC, and P=0.009 for IL-6 at 4 hours). TNFα concentrations in the BAL were significantly elevated 2 hours after LPS injection, but not at 4 hours (P= 0.048 at 2 hours, P= 0.630 at 4 hours). (D) BAL total mononuclear cells (P=0.658) and neutrophils 4 h after LPS injection (P=1). Lung MPO activity was increased from baseline at 4 h following tail vein LPS injection (P<0.001). Data represent 3 independent experiments, N=9-12 animals/group.

Figure 2. BAL cytokines, airspace neutrophil counts and lung MPO activity in endotoxemic mice 4 hours following transfusion of 1-2 d stored RBC. (A) RBC transfusion increased BAL MIP-2 compared to Hextend controls (P=0.009). BAL KC, IL-6 and TNFα were not increased following RBC transfusion (P=0.232, P=0.281 and 0.955 respectively). (B) Total BAL mononuclear cell counts were not different between the two groups (P = 0.601). RBC transfusion increased airspace neutrophils when compared with Hextend® (2433 v 255, P<0.001). Lung MPO activity was similar between the two groups. (C and D) GR-1 staining of lung sections from 1-2 d RBC transfused animals show increased interstitial and airspace neutrophils compared with
Hextend® (HEX) transfused controls. Magnification 400x. Data are representative of 3 independent experiments, N=9-12 animals/group.

**Figure 3. BAL cell counts, lung MPO activity and microvascular permeability 4 hours following transfusion of unwashed 10 d RBC in endotoxemic mice.** (A) Transfusion of 10 d RBC increased airspace neutrophil recruitment when compared with < 1d RBC (P=0.015). Lung MPO activity was similar between the two groups. (B) Transfusion of 10 d RBC increased lung microvascular permeability when compared with < 1d RBC (P=0.021). Data are representative of 3 individual experiments, N= 9 animals in each group.

**Figure 4. BAL cell counts, lung MPO activity, microvascular permeability and BAL cytokines 4 hours following transfusion of washed 10 d RBC in endotoxemic mice.** Washing erythrocytes immediately prior to transfusion did not abrogate lung injury after transfusion of 10 d RBC. (A) Transfusion of washed 10 d RBC increased airspace neutrophil recruitment when compared with RBCs stored for < 1d (P=0.007). (B) Transfusion of washed 10 d RBC increased lung microvascular permeability when compared with < 1d RBC (P=0.039). (C) BAL MIP-2 and KC were increased 4 hours following transfusion of washed 10 d RBC (P=0.021 and P=<0.001, for MIP-2 and KC respectively). Data are representative of 2 independent experiments, N= 8 animals in each group.
**Figure 5. Transfusion of WT or Duffy KO RBCs during endotoxemia.** Mice were transfused with 1-2 d KO or WT RBC 2 hours following LPS administration. (A) Transfusion of KO RBC increased BAL cytokines 4 hours after transfusion when compared with WT RBC transfusion (P = 0.008, 0.022, 0.022 and 0.013 for MIP-2, KC, IL-6, and TNF α respectively). (B) Total BAL mononuclear cells were similar between the KO and WT RBC transfused animals (P = 0.233). There was a significant increase in airspace neutrophil counts in the KO RBC transfused animals when compared with WT RBC transfused animals, P = <0.001. (C) Transfusion of KO RBC increased lung microvascular permeability when compared with transfusion of WT RBCs, P=0.034. Data are representative of 3 individual experiments, n=12 in each experimental group.

**Figure 6. Chemokine scavenging function of murine erythrocytes.** (A) 1 x 10^8 erythrocytes with differing storage times were incubated with 2 nM CCL2. CCL2 concentrations in supernatant increased with RBC storage time, P<0.001. (B) 1 x10^8 WT or KO erythrocytes were incubated with 2 nM CCL2. For chemokine incubated with WT erythrocytes, CCL2 concentrations increase in the supernatant and decrease in the lysate over time, (+) P= 0.006 and P=0.011 for WT supernatant and lysates respectively, P=NS for KO. There were significant differences in CCL2 between WT and KO erythrocytes (* indicates P=0.027 and P=0.012 for supernatant from 11 and 21 day old erythrocytes, P=0.057 from 17 days old erythrocytes; lysates P<0.001 for all days). (C) 1 x10^8 WT or KO erythrocytes were incubated with 2 nM MIP-2 for 30 minutes. P<0.001 for WT erythrocytes and P=0.102 for KO erythrocytes. There were significant differences in supernatant MIP-2 between WT and KO erythrocytes that were stored for 1, 2, 9 or 12
days (P = 0.002, P = 0.019, P = <0.001, and P = 0.002 respectively), but not when erythrocytes were stored for 16, 23 or 26 days (P = 0.091, P= 0.200, P = 0.642 respectively).

**Figure 7. Chemokine scavenging function and Duffy Antigen expression in stored human erythrocytes.** (A) 1 x 10^8 erythrocytes from a volunteer were incubated with 2 nM CCL2 for 30 min, P=0.002 for supernatant and P=0.021 for cell lysates. (B) 1x 10^8 erythrocytes from PRBC units were incubated with 2nM CXCL1 or CXCL8. Increased soluble CXCL1 concentrations in 27 d RBC compared to 12 d RBC, P=0.008; no difference in soluble CXCL8 concentrations, P=0.999. (C) Duffy antigen expression on erythrocytes from banked blood (11, or 31 days old) compared with fresh erythrocytes isolated from healthy volunteers. (D) Storage time correlates with reduction in Duffy antigen expression on banked erythrocytes, P=0.021.
Figure 1
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>MIP-2</th>
<th>KC</th>
<th>IL-6</th>
<th>TNFα</th>
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B

<table>
<thead>
<tr>
<th></th>
<th>BAL MONONUCLEAR ($10^5$)</th>
<th>BAL PMN ($10^5$)</th>
<th>MPO ACTIVITY</th>
<th>LUNG</th>
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<tbody>
<tr>
<td>HEX</td>
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<td>LPS + 1-2d RBC</td>
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C

D

HEX

RBC
Figure 3
Figure 4

A

B

C

Washed RBCs

Washed RBCs

Washed RBCs

BAL MONONUCLEAR (x10^5)

BAL MONONUCLEAR (x10^5)

BAL TOTAL PROTEIN (ug/mL)

BAL x10^2 (pg/mL)

BAL x10^2 (pg/mL)

MIP-2

MIP-2

KC

KC

LPS + <1d RBC

LPS + <10d RBC

LPS + <1d RBC

LPS + <10d RBC

LPS + <1d RBC

LPS + <10d RBC

* statistically significant difference from control
Figure 5

A

![Graph showing cytokine levels in BAL fluid.](Image)

- MIP-2
- KC
- IL-6
- TNFα

LPS + WT
LPS + KC

B

![Graph showing cellular and MPO activity in BAL fluid.](Image)

- BAL mononuclear (x10^5)
- BAL PMN (x10^4)
- MPO activity / g LUNG

C

![Graph showing total protein levels in BAL fluid.](Image)

- BAL total protein (μg/mL)
Figure 6
Figure 7

A

Soluble CCL2 (pg/mL)

Supernatant | Lysate

1 d RBC | 13 d RBC

B

Soluble CXCL1 (pg/mL)

Soluble CXCL8 (pg/mL)

12 d RBC | 27 d RBC

C

D

MFI

RBC STORAGE TIME (DAYS)

Duffy Negative | Isotype Control

Fresh RBC | 11 d RBC | 31 d RBC

10^0 10^1 10^2 10^3 10^4 10^5

Fy6

1809 1357 905 452 0

0 20 40 60 80 100 120 140 160 180
Loss of red cell chemokine scavenging promotes transfusion related lung inflammation

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