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Leukoreduction of Packed RBCs Attenuates Proinflammatory Properties of Storage-Derived Microvesicles

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Abstract

Background—Leukoreduction prior to packed red blood cell (pRBC) storage is not a universally accepted practice. Our laboratory has previously shown that microvesicles (MV) accumulate in pRBC units during storage and play an important role in lung injury after resuscitation. Currently, the effect of leukoreduction on MV formation in stored pRBC units is unknown. In the present study, we investigated the hypothesis that leukoreduction of pRBC units prior to storage would attenuate the production of MVs and decrease pulmonary inflammation after hemorrhage and resuscitation.

Methods—Leukoreduced and non-leukoreduced pRBC units were prepared from human donors and C57/Bl6 mice and stored for up to 42 days or 14 days, respectively. At intervals during storage, MVs were isolated from pRBC units, quantified, and characterized based on size, morphology, and levels of proinflammatory cytokines. In additional experiments, mice underwent controlled hemorrhage followed by resuscitation with normal saline (NS) with or without equal numbers of MV isolated from leukoreduced or non-leukoreduced stored mouse pRBC. Histologic lung sections were evaluated for the presence of tissue edema and inflammatory cells.

Results—For both human and mouse pRBC, the number of MV significantly increased throughout the storage period. There were significantly fewer MV present in leukoreduced units. The average MV size significantly increased over time and was similar between groups. Levels of IL-1 α , RANTES, and MDC were lower in MV from leukoreduced pRBC units as compared to MV from non-leukoreduced units. Hemorrhaged mice resuscitated with NS with the addition of

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MV from leukoreduced pRBC demonstrated significantly less pulmonary edema and inflammatory cell recruitment as compared to those resuscitated with NS with the addition of MV from non-leukoreduced pRBC.

Conclusions—Pre-storage leukoreduction of pRBC units reduces the formation and proinflammatory properties of MV, which in turn decreases lung injury secondary to MV from stored pRBC units after hemorrhage and resuscitation.

Keywords

Blood storage; microparticles; lung inflammation; MDC/CCL22; IL-1; RANTES

INTRODUCTION

Following severe hemorrhage, resuscitation with blood products is the primary treatment for the restoration of circulating blood volume, replacement of coagulation factors, and improvement of oxygen-carrying capacity. Resuscitation with large volumes of stored blood products, however, is associated with several complications, including sepsis, coagulopathy, multiple organ dysfunction and death.^{1–6} The age of packed red blood cell (pRBC) units has been identified as an independent, risk factor of morbidity and mortality,^{5,7,8} and therefore transfusions using fresher blood products may be the preferred treatment for many patient populations.

The mechanisms underlying the deleterious clinical consequences of pRBC storage are not clearly understood, but recent efforts have provided additional insight into the “red blood cell storage lesion”.⁹ During storage, red blood cells (RBC) undergo a series of structural and biochemical changes that undermine RBC viability, result in the release of potentially hazardous products, and ultimately reduce the effectiveness of blood transfusions. In addition to changes in cell shape and membrane integrity, factors such as microvesicle (MV) formation, inhibition of nitric oxide signaling, and loss of red cell chemokine scavenging are believed to also contribute to the clinical complications evident with transfusion of older blood.^{9–13}

Antonelou et al. have previously demonstrated that leukoreduction significantly alleviates many of the storage-related modifications of RBC senescence and oxidative stress suggesting that passenger leukocytes within non-leukoreduced red cell units counter the structural and functional integrity of RBC.¹⁴ Although there is growing rationale for implementation of pre-storage leukoreduction,^{15–17} the practice is not a universal standard for blood banks within the United States or internationally.

We recently demonstrated that MV formed during the storage of non-leukoreduced pRBC units contribute to neutrophil priming and activation.¹⁰ Resuscitation of hemorrhaged mice with non-leukoreduced storage-derived MV led to substantially increased pulmonary edema and neutrophil recruitment suggestive of an association between non-leukoreduced RBC-derived MV and lung injury following transfusion.¹⁰ Given the possible benefits of leukoreduction for alleviating storage-derived RBC changes, the goals of the current study were to analyze MV formation during storage of leukoreduced and non-leukoreduced

pRBCs and to evaluate the role of leukoreduction in the development of MV-mediated pulmonary injury following hemorrhage and resuscitation.

MATERIALS AND METHODS

Reagents

Citrate phosphate double dextrose (CP2D; 257.6 mmol/L glucose, 105 mmol/L citrate-citric acid, 18.5 mmol/L monosodium phosphate, pH 5.7) was prepared as anticoagulant. AS-3 (Nutricel; 55.5 mmol/L glucose, 70.1 mmol/L sodium chloride, 20 mmol/L monosodium phosphate, 12 mmol/L citric acid, 2.2 mmol/L adenine, pH 5.8) was used as red cell storage media.

Mice

Male C57/Bl6 mice weighing 21–30 grams were purchased from Harlan Laboratories (Indianapolis, IN), fed standard laboratory diet and water ad libitum and acclimated for 1 week in a climate controlled room with a 12 h light-dark cycle. Male mice were used to avoid variability caused by hormonal cycles in females. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Blood Banking

Murine blood was collected and stored as previously described.¹⁸ Following anesthetization with intraperitoneal pentobarbital (0.1 mg/g body weight), blood was collected via cardiac puncture into CP2D-coated syringes. Whole blood was then mixed with CP2D at a ratio of 1:7, mixed gently and pooled. After IRB approval and patient consent, human blood was collected from healthy donors using a standard venipuncture protocol and anti-coagulated with CP2D.

For leukoreduction, a portion of the whole blood collected was passed through a Pall neonatal leukoreduction filter (Pall Biomedical Products Company, East Hills, NY). Units of leukoreduced blood and non-leukoreduced whole blood were then centrifuged at 100g for 10 min and the platelet-rich plasma (PRP) removed with gentle pipetting. Packed RBCs were obtained following centrifugation at 1000g for 15 min and resuspended in AS-3 storage buffer at a ratio of 2:9 based on the initial volume of blood prior to removal of the PRP. For characterization studies, leukoreduced and non-leukoreduced pRBC were divided into aliquots and analyses conducted on days 0, 7, 14, 28 and 42 for human samples (n=3 per timepoint) and days 0, 7 and 14 for murine samples (n=4 per timepoint). These time points were chosen based on our previous work that establishes a 14 day storage period for murine pRBC that is comparable to the current FDA-approved 42 day storage period from human blood.¹⁸

MV Isolation and Characterization

At each time point, aliquots of stored pRBC were centrifuged at 1500g for 10 min and the MV-rich supernatant collected as described previously.^{10,19} Residual erythrocytes were removed by a subsequent 1500g centrifugation, and then a portion of MV in the supernatant was counted using flow cytometry as described below. The remaining MV sample was

pelleted at 16,000g for 20 min, resuspended in double filtered PBS, and analyzed with either dynamic light scattering (DLS) or transmission electron microscopy (TEM). For cytokine analyses, MV isolated from mouse pRBC stored for 7 days were flash-frozen and cytokine levels were quantified by multiplex ELISA (Quansys Biosciences, Logan, UT) according to the manufacturer's instructions. The multiplex ELISA contained a panel of 20 cytokines, including granulocyte macrophage colony-stimulating factor, interferon γ , interleukin 1 α (IL-1 α), IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, keratinocyte chemoattractant, macrophage-derived chemokine (MDC), macrophage inflammatory protein 1 α (MIP-1 α), MIP-2, monocyte chemoattractant protein 1, regulated on activation, normal T cell expressed and secreted (RANTES), thymus- and activation-regulated chemokine (TARC), and tumor necrosis factor α .

For MV counting, 25 μ L of MV-rich supernatant was added to 275 μ L of double-filtered PBS. The number of events in 20 μ L of sample was recorded using a Beckman Coulter XL flow cytometer (BD Biosciences, San Jose, CA). Following subtraction of background events in PBS alone, final values were reported as MV per μ L of sample.

Dynamic light scattering (DLS) was used to measure the size distribution of storage-derived MV. At each time point, pelleted MV were resuspended in filtered PBS and analyzed with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

Transmission Electron Microscopy

For transmission electron microscopy (TEM) processing, formvar/carbon grids (Electron Microscopy Sciences, Hatfield, PA) were wet with 0.1% BSA solution for 5 min. Excess fluid was wicked away and 20 μ L of MV sample was added for 10 min. Excess sample was wicked away, and the MV were stained with 10 drops of 1% uranyl acetate and air dried. Samples were viewed at 80 kV with a Hitachi H7650 electron microscope equipped with an AMT V600 digital camera.

Murine Model of Hemorrhage and Resuscitation

Murine hemorrhage and resuscitation was carried out in a similar fashion as described previously.²⁰ Mice underwent femoral artery cannulation followed by a 10 min equilibration period, a 60 min period of pressure-targeted hemorrhage and a 20 min period of fluid resuscitation. During the hemorrhage period, blood was withdrawn in a controlled manner via the femoral artery until a systolic blood pressure of 25 mmHg was achieved. Mice were maintained at a pressure of 25 mmHg $\pm$ 5 mmHg for 60 min followed by resuscitation to a systolic blood pressure of 80 mmHg $\pm$ 5 mmHg for 20 min. Sham animals underwent identical femoral artery cannulation and monitoring for 90 min, but were neither hemorrhaged nor resuscitated. Mice were then decannulated, monitored and sacrificed at 4 h. Each experimental and control group consisted of four animals per treatment. For resuscitation experiments, leukoreduced and non-leukoreduced pRBC units were acquired and stored under similar conditions as described above. MV were isolated after 14 days of storage, quantified and reconstituted in normal saline. Equal numbers of either leukoreduced or non-leukoreduced MV were delivered in a 100 μ L bolus immediately after hemorrhage followed by resuscitation with normal saline as needed for the remainder of the resuscitation

period. Normal saline was used for MV delivery and for resuscitation in order to allow focused study of the effects of MV on lung injury. Our previous work indicates that lung injury after resuscitation with older pRBC units is mediated by MV from those units.¹⁰

Statistical Analyses

Data were assessed for normality and equal variances and analyzed using a Student's *t*-test, analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc tests or Kruskal-Wallis test. Normally distributed data are reported as mean + standard error of the mean (SEM). Statistical analyses were performed using SigmaPlot 11 software (Systat Software, Chicago, IL).

RESULTS

The formation of MV increased during the storage of both mouse and human pRBC (Figure 1). The greatest number of MV were observed in non-leukoreduced units of mouse pRBCs after 14 days of storage (Figure 1A). Pre-storage leukoreduction of mouse pRBCs significantly attenuated the number of storage-derived MVs. Likewise, significantly more storage-derived MV were observed in human pRBC units at the time of expiration (day 42), and MV formation was reduced by pre-storage leukoreduction (Figure 1B).

The physical characteristics of the MV population formed during storage were assessed using DLS and TEM. Based on the size distribution profile, a primary MV population of approximately 200 nm existed for both mouse and human MV (Figures 2a and 2b, respectively). At early time points, a smaller ~50 nm subpopulation was also present. This population disappeared by day 7 for mouse MV and by day 14 for human MV. Thus, for both mouse and human pRBC units, the average MV size was approximately 75 nm at storage day 0, and the size of MV increased significantly during pRBC storage (Figure 2C and 2D, respectively). MV generated during storage of mouse pRBC had a broader size distribution at day 14 with an average size of approximately 350 nm at the time of expiration. MV formed during human pRBC storage maintained an average size of about 200 nm starting at day 14 of storage until the time of expiration (day 42). In general, leukoreduction had no significant effect on the size of MV for either mouse or human pRBC. However, MV derived during the storage of human leukoreduced pRBC were slightly smaller at days 7 and 14 compared to non-leukoreduced MV.

The morphology of isolated storage-derived MV was assessed at the end of the storage period. No differences were observed between leukoreduced and non-leukoreduced MV for either mouse (Figures 3A and 3B) or human pRBC (Figures 3C and 3D) pRBC units.

In order to determine aspects of potential pro-inflammatory properties of MV, chemokine levels were measured in MV isolated from murine pRBC units. Of the 20 chemokines and cytokines measured, significant differences were found in the levels of IL-1 α , RANTES, and MDC in non-leukoreduced as compared to leukoreduced MV (Figure 4). For each of these cytokines, pre-storage leukoreduction of pRBC significantly reduced the levels present in MV formed during the storage period.

In order to determine the effect of pre-storage leukoreduction on lung injury after resuscitation with aged pRBC units, mice underwent hemorrhage followed by resuscitation with normal saline with the addition of MVs isolated from either non-leukoreduced or leukoreduced pRBC units. In this *in vivo* model of hemorrhage and resuscitation, administration of non-leukoreduced MV at the time of resuscitation significantly increased pulmonary edema and recruitment of inflammatory cells (Figure 5).

DISCUSSION

In the present study, we demonstrate that pre-storage leukoreduction attenuates the formation and proinflammatory properties of storage-derived MV. Leukoreduction reduced the number of MV of both human and mouse pRBC shed during storage without affecting the physical properties (i.e. size and morphology) of the primary MV population at the time of pRBC expiration. For mouse pRBC, leukoreduction also significantly reduced the levels of inflammatory cytokines that were present in MV that formed during storage. Consequentially, administration of MV derived from stored leukoreduced pRBC to hemorrhaged mice at the time of resuscitation resulted in significantly less pulmonary edema and inflammatory cell recruitment as compared to resuscitation with fluid containing MV from non-leukoreduced pRBC. This work supports the beneficial effect of pre-storage leukoreduction of pRBC units in order to reduce the formation of (and pro-inflammatory mediators in) storage-derived MV that contribute to lung injury after hemorrhage and resuscitation with stored blood.

MV have been previously shown to accumulate during pRBC storage and to cause proinflammatory activity after transfusion, although the exact mechanisms responsible for post-transfusion complications remain unclear.^{10,21–24} Data from our lab suggest that storage-derived MV contribute to neutrophil priming and activation and to transfusion-related lung injury.¹⁰ Other groups have demonstrated that the neutrophil priming activity of stored pRBC is present in both MV-rich and MV-poor fractions,²¹ and others reported that soluble mediators outside of MV (most likely protein-bound RNA) are responsible for suppression of monocyte function *in vitro*.²⁵ The current study confirms that the pRBC fraction containing MV formed during the storage of non-leukoreduced pRBC possesses pro-inflammatory activity, which exacerbates pulmonary injury after hemorrhage and resuscitation. Furthermore, our data demonstrate that leukoreduction of RBC prior to storage attenuates the formation of this proinflammatory MV population.

In non-leukoreduced pRBC units, passenger leukocytes have been implicated for contributing to transfusion-related immunomodulation through the production of bioactive substances that accumulate during storage. Leukoreduction is a proposed method to mitigate the deleterious effects of stored blood by eliminating leukocytes and their proinflammatory mediators. In the current study, leukoreduction resulted in a significant reduction in MV levels of the proinflammatory mediators IL-1 α , MDC and RANTES. Data from our and other laboratories have shown that these mediators are associated with lung inflammation and suggest that the higher levels of IL-1 α , MDC and/or RANTES contributed to the pulmonary edema and cell infiltration observed after resuscitation with MV generated during the storage of non-leukoreduced pRBC units.^{26–28}

IL-1 α is a key regulator of effector molecules and chemokines responsible for the recruitment and trafficking of monocytes and neutrophils to sites of injury. Exacerbated IL-1 α release caused by trauma is central to the progression of lung injury.^{29,30} Conversely, RANTES is characterized as a chemoattractant for monocytes, T-lymphocytes and eosinophils, and its role in acute lung inflammatory injury is less clear. Whereas RANTES had no effect on the development of lung injury caused by intrapulmonary deposition of IgG immune complexes,³¹ other groups have reported a correlation between RANTES levels and lung neutrophilia.^{28,32} MDC is a chemoattractant for CCR4-expressing lymphocytes, monocytes, monocyte-derived dendritic cells, and natural killer cells.³³ We have previously shown that MDC is elevated in mice after hemorrhage and resuscitation²⁰ and that MDC acts a critical mediator of hemorrhage/resuscitation-induced lung injury.²⁶ Inhibition of MDC significantly reduced pulmonary levels of chemotactic cytokines and inflammatory cell infiltration, whereas administration of recombinant MDC to hemorrhaged mice exacerbated pulmonary edema and inflammation.²⁶ Thus, the increased level of MDC present in MV derived from non-leukoreduced blood is likely a key factor in the pulmonary inflammatory response observed *in vivo*.

Our data suggest that leukoreduction did not affect the morphology of MVs shed from pRBC units during storage. Overall, we found that the MV generated during pRBC storage had similar morphological features and size. Human MV formed in leukoreduced pRBC were slightly smaller in size than non-leukoreduced MV at days 7 and 14, although this difference was not observed at later time points. The reasons for the MV size differences at early storage time points and whether MV size effects their proinflammatory properties requires further investigation as our cytokine analyses and *in vivo* studies utilized MV with similar size. Thus, the post-transfusional effect of leukoreduced and non-leukoreduced MV are most likely based on biochemical properties, such lipid composition or presence of inflammatory mediators.

CONCLUSIONS

The formation of MV is a hallmark characteristic of the RBC storage lesion that appears to contribute to inflammation-based complications that occur following transfusion with stored blood products. This study demonstrates that leukoreduction attenuates the formation and proinflammatory properties of storage-derived MV by reducing levels of chemoattractant cytokines responsible for pulmonary edema and inflammation after resuscitation. These findings have important clinical implications for attenuating transfusion-related organ dysfunction, including acute lung injury, that contribute to secondary complications and increased mortality after trauma.

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Figure 1. Microvesicle (MV) formation in mouse and human packed red blood cells (pRBCs) increases with storage time and is decreased by pre-storage leukoreduction

In both (A) mouse and (B) human pRBCs, leukoreduction attenuated storage-derived MV formation at the end of the storage period. (●) Non-leukoreduced; (○) Leukoreduced.

* $p < 0.001$ vs non-leukoreduced previous time points; + $p < 0.05$ vs leukoreduced previous time points; † $p < 0.001$ vs leukoreduced day 14 time point.

Figure 2. Microvesicles (MV) formed during storage of packed red blood cells (pRBCs) are larger in size compared to MV present at storage day 0
 Representative intensity plots obtained from dynamic light scattering analyses of MV formed during the storage of (A) mouse and (B) human packed red blood cells (pRBC). From these data, the average MV size of (C) mouse and (D) human MV was determined. (●) Non-leukoreduced; (○) Leukoreduced. * $p < 0.01$ and ** $p < 0.001$ vs non-leukoreduced Day 0; + $p < 0.01$ vs ++ $p < 0.001$ vs leukoreduced Day 0.

Figure 3. Transmission electron micrographs of storage-derived microvesicles (MV)
Representative micrographs of MV isolated from mouse (A) non-leukoreduced packed red blood cells (pRBC) and (B) leukoreduced pRBC after 14 days of storage. MV were also isolated from human (C) non-leukoreduced and (D) leukoreduced pRBC after 42 days of storage. Magnification is 100,000 $\times$. Scale bars are 100 nm.

Figure 4. Microvesicles from stored non-leukoreduced pRBC (nLR MV) have increased levels of IL-1 α , MDC and RANTES compared to microvesicles from stored leukoreduced pRBC (LR MV). Cytokine levels present in isolated MV were measured by ELISA after 7 days of mouse pRBC storage. * $p < 0.05$ vs non-leukoreduced.

Figure 5. Leukoreduction decreases pulmonary inflammation caused by resuscitation with microvesicles from stored non-leukoreduced pRBC (nLR'd MV)

Representative micrographs of lung tissue stained with hematoxylin and eosin (**A–D**) and anti-mouse Ly-6B.2 (**E–H**) for inflammatory cell identification. Mice resuscitated with normal saline (NS) that contained MV isolated from nLR'd mouse pRBC stored for 14 days had substantial (**D**) interstitial tissue edema and (**H**) cell infiltration compared to (**A,E**) sham mice, (**B,F**) mice resuscitated with only NS and (**C,G**) mice resuscitated with NS plus MV isolated from leukoreduced (LR'd) mouse pRBC. (**I**) Quantification of positively-stained cells confirmed the effect of leukoreduction on decreased inflammatory cell recruitment caused by storage-derived MV. * $p < 0.005$ vs Sham and ** $p < 0.001$ vs all groups, $n = 4$ per group.