Anesthesiology 2010; 113:92–103

Supernatant of Aged Erythrocytes Causes Lung Inflammation and Coagulopathy in a "Two-Hit" *In Vivo* Syngeneic Transfusion Model

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ABSTRACT

Background: Transfusion of erythrocytes is associated with increased morbidity in certain patient groups. Storage time of erythrocytes may contribute to respiratory complications. Using a syngeneic *in vivo* transfusion model, we investigated whether transfusion of stored rat erythrocytes causes lung injury in healthy and in lipopolysaccharide-primed rats in a "two-hit" model of lung injury.

Methods: Rats were infused with aged rat erythrocytes (14 days of storage) and washed aged erythrocytes or supernatant of aged erythrocytes. Controls received fresh rat erythrocytes (0 days of storage) or saline. In the "two-hit" model of lung injury, lipopolysaccharide was used as a "first hit" before transfusion. Rat and control human erythrocyte products were analyzed for lysophosphatidylcholine accumulation.

Results: In healthy rats, transfusion of aged erythrocytes caused mild pulmonary inflammation but no coagulopathy. In lipopolysaccharide-pretreated rats, transfusion of aged erythrocytes augmented lung injury by inducing coagulopathy, both in the pulmonary and systemic compartment, when compared with transfusion with fresh erythrocytes. When transfused separately, supernatant of aged erythrocytes, but not washed aged erythrocytes, mediated coagulopathy in the "two-hit" model. Analysis of the supernatant of aged erythrocytes (rat and human) showed no lysophosphatidylcholine accumulation.

Conclusions: Transfusion of aged erythrocytes induces lung injury in healthy rats. In a "two-hit" model, injury induced by aged erythrocytes was characterized by coagulopathy and was abrogated by washing. Washing of aged erythrocytes may decrease pulmonary complications in patients with an inflammatory condition who are exposed to a blood transfusion.

What We Already Know about This Topic

- Transfusion of aged erythrocytes is associated with transfusion-related acute lung injury, but the mechanisms for this injury are unknown

What This Article Tells Us That Is New

- Transfusion of aged erythrocytes in normal rats with earlier infusion of lipopolysaccharide resulted in lung inflammation and coagulopathy
- This effect occurred with the supernatant, but not with washed cells, suggesting that washing may prevent this injury

TRANSFUSION of erythrocytes has increased in the past years. This increase may be explained by an aging population and by evolving surgical and medical procedures.[1] Annually, almost 14,000,000 erythrocyte units are transfused in the United States.[2] However, it is increasingly recognized that transfusion of erythrocytes is associated with morbidity and mortality in certain patient populations, including critically ill, postoperative, and trauma patients.[3]

◆ This article is accompanied by an Editorial View: Shander A, Javidroozi M: A reductionistic approach to aged blood. ANESTHESIOLOGY 2010; 113:1–3.

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Received from the Laboratory of Experimental Intensive Care and Anesthesiology, Academic Medical Center, Amsterdam, The Netherlands. Submitted for publication November 6, 2009. Accepted for publication February 4, 2010. Support was provided solely from institutional and/or departmental sources.

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The age of erythrocytes has been implicated as a causative factor in transfusion-related complications.[4–10] In particular, transfusion of aged erythrocytes is associated with respiratory complications.[8,11] The mechanism linking adverse outcomes with erythrocyte storage remains unclear. A decreased deformability capacity and an increased adhesiveness of the aged erythrocyte, donor white blood cells, and soluble factors such as cytokines and bioactive lipids (*i.e.*, lysophospatidylcholines) have all been suggested to mediate adverse effects.[12–21]

Aged blood products have been associated with the occurrence of transfusion-related acute lung injury (TRALI) in the clinical setting[18] and have been used to induce TRALI in "two-hit" animal models.[17,22] In the "two-hit" hypothesis, TRALI is the result of endothelial activation, caused by an underlying inflammatory condition (*e.g.*, pneumonia or sepsis), resulting in priming of the pulmonary neutrophils. This "first hit" is followed by activation of the primed neutrophils caused by the "second hit" (transfusion of a blood product), resulting in activation of the primed neutrophils, with subsequent endothelial damage and capillary leak, leading to pulmonary edema. Coagulopathy and decreased fibrinolysis are distinctive features of acute lung injury due to other causes,[23,24] contributing to morbidity and mortality.[25,26] As the endothelium initiates and regulates coagulation,[27] it can be hypothesized that coagulopathy may also play a role in TRALI. However, data on coagulation during TRALI are absent.

The "two-hit" hypothesis has been proposed as a mechanism to explain why critically ill patients, who frequently suffer from an inflammatory condition, are susceptible to a TRALI reaction.[28–30] Because transfusion is associated with adverse outcome, at least in certain patient groups, including trauma patients and the critically ill,[11,31–37] it is important to study pathways of disease in models that represent the clinical situation. Current TRALI models investigating the role of storage time of blood products are limited by cross-species design and modification of transfusion protocols.[17,22,38] Currently, no clinically relevant "in species" transfusion model using a clinical preparation protocol has confirmed the hypothesis that aged erythrocytes contribute to lung injury.[39] We investigated the effect of aged rat erythrocytes on lung inflammation and coagulation in a syngeneic *in vivo* erythrocyte transfusion model in healthy rats. Similar to a model of patients with an underlying inflammatory condition, the effect of aged erythrocytes was also studied in a "two-hit" model of lung injury, using lipopolysaccharide-primed rats.[17,40] In addition, we evaluated whether washing of erythrocytes influenced the development of lung injury inflicted by transfusion.

Materials and Methods

The Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, The Netherlands and the Medical Ethical Committee of Sanquin Blood Bank Foundation approved all experiments. All animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care, and handling of laboratory animals.

Preparation of Rat Erythrocyte Products

Male Sprague–Dawley rats (>250 g; Harlan, The Hague, The Netherlands) were used to obtain blood. Rats were anesthetized with an intramuscular injection of 45 mg/kg ketamine (Eurovet, Bladel, The Netherlands) and 0.25 mg/kg medetomidine (Novartis, Arnhem, The Netherlands). Blood was collected from the inferior vena cava in a syringe containing 1.25 ml citrate–phosphate–dextrose (Fersenius HemoCare GmbH, Bad Homburg, Germany). Approximately 8–10 ml blood could be obtained from a single rat. Blood of five rats was pooled for component preparation. Before pooling, cross-matching was carried out to ensure compatibility.

Blood was handled and stored according to national standards for human blood (Sanquin Blood Supply Foundation, Amsterdam, The Netherlands), with minor changes to adapt for the smaller volumes. After overnight storage at room temperature, blood was centrifuged for 10 min at 1,892*g* and 20°C. Plasma was removed, and the buffy coat was separated from the packed erythrocytes. Saline–adenine–glucose–mannitol was added to the erythrocytes up to a hematocrit of 55–60%. The final products were stored in 50-ml Falcons at 4°C, which were partly open.

Preparation of Washed Aged Erythrocytes and Supernatant Rat Erythrocyte Products

After 14 days of storage, rat erythrocyte products were separated into washed erythrocytes and supernatant. To obtain as much as possible soluble factors from the product, 0.9% NaCl was added to rat erythrocyte products before centrifugation. The erythrocyte–NaCl mixture was centrifuged for 15 min at 1,500*g* and 4°C. For the final supernatant used in the experiment, 0.9% NaCl was added to the supernatant up to the original volume of the rat erythrocyte product. The erythrocytes were washed using saline–adenine–glucose–mannitol (1:1) and centrifuged for 15 min at 1,250*g* and 4°C. The supernatant was removed, and the saline–adenine–glucose–mannitol was added to the erythrocytes up to the original volume of the rat erythrocyte product.

In Vivo *Erythrocyte Transfusion Models*

Male Sprague–Dawley rats (275 g) fed with a regular diet were weighed and anesthetized with 50 mg/kg pentobarbital intraperitoneally. The tail vein was cannulated with a 24-gauge venflon (Vasofix Certo; B.Braun, Meisungen, Germany), and blood was aspirated to verify intravascular placement and to remove 0.5 ml blood for cross-matching and baseline measurements. A 10% circulating volume transfusion was administered more than 30 min using an infusion pump (Harvard Pump 11; Harvard Apparatus, Holliston, MA).

Animals were randomized by an independent researcher into three groups (n = 6 per group) to receive transfusion with 0.9% NaCl, fresh erythrocytes, or erythrocytes stored for 14 days (aged erythrocytes). A storage time of 14 days was chosen because pilot experiments showed that rat erythrocytes stored for 14 days showed storage-related changes that were comparable with those found in previous studies that compared human erythrocytes stored for 28–35 days with rat erythrocytes stored for 14 days.[41] Rats were placed back in their cages to recover and were killed 6 h after transfusion. In a separate set of experiments, animals were transfused with washed aged erythrocytes or with supernatant of washed aged erythrocytes. For the experiments in the "two-hit" erythrocyte transfusion model, rats received 2 mg/kg lipopolysaccharide (from *Salmonella enteritidis*; Sigma; St. Louis, MO) intraperitoneally 2 h before transfusion. This dose has been used before as a "first hit" in TRALI models, including a model using aged human erythrocytes, and was shown to result in sequestration of neutrophils in the lungs.[17,22,40] Controls received saline (equal volume).

Blood and Tissue Sampling

After anesthesia with ketamine and medetomidine as described, blood was collected from the inferior vena cava in citrated (0.109 M) vacutainer tubes for analysis and blood culture. The right lung was ligated, and the left lung was lavaged three times with 2 ml saline. After lavage, lungs were weighed and homogenized using a tissue homogenizer (Biospec Products, Bartlesville, OK). For cytokine and chemokine measurements, lung homogenates were diluted 1:1 in lysis buffer (150 nmol/l NaCl; 15 mmol/l Tris; 1 mmol/l $MgCl_2$–H_2O; 1 mmol/l $CaCl_2$; 1% Triton X-100; and 100 μg/ml pepstatin A, leupeptin, and aprotinin). The right lung was fixed in 4% formalin and embedded in paraffin for histopathology examination. Four-micrometer sections were stained with hematoxylin–eosin and analyzed by two researchers who were blinded for group identity. A histology scoring system was used as previously described.[42] In short, the following parameters were scored on a scale of 0–4: (1) interstitial inflammation, (2) endothelialitis, (3) bronchitis, (4) edema, (5) thrombus, and (6) pleuritis. The histology score was expressed as the sum of the score for all parameters.

Assays

Thrombin–antithrombin complexes (TATc; Behring, Marburg, Germany) and fibrin degradation products (Asserachrom D–Di; Diagnostica Stago, Asnières–sur–Seine, France) were measured using enzyme-linked immunosorbent assay. The activities of plasminogen activator and plasminogen activator inhibitor-1 were measured by automated amidolytic assays.[43] Tumor necrosis factor, interleukin-6, and cytokine-induced neutrophil chemoattractant-3 were measured by enzyme-linked immunosorbent assay according to instructions from the manufacturer (R&D Systems, Abingdon, United Kingdom), and the detection limit was 62.5, 31.25, and 125 pg/ml, respectively.

Storage-related Biochemical Changes in Rat Erythrocytes

erythrocyte samples were collected at the indicated time intervals and analyzed for pH, potassium, sodium, glucose, and lactate using a Rapidlab 865 blood gas analyzer (Siemens Medical Solutions Diagnostics, Breda, The Netherlands). Cell counts for leukocytes and erythrocytes were done with an Advia 2120 hematology counter, using special software for counting animal blood samples (Siemens Medical Solutions Diagnostics). Supernatants were prepared by centrifugation for 10 min at 14,500*g* at 4°C to remove cells and acellular debris. Aliquots of supernatants were stored at −80°C for analysis of lysophosphatidylcholine, phosphatidylcholine, and cytokine levels.

Lipid Extraction and Lysophosphatidylcholine and Phosphatidylcholine Measurement

Lipid extraction of supernatant from stored erythrocyte supernatant was performed using Bligh and Dyer method. In short, 3 ml $CHCl_3$:MeOH (1:2) was added to 100 μl sample and 100 μl internal standard solution (2.5 nM lysophosphatidylcholine 14:0, and 10 nM phosphatidylcholine 28:0). Seven hundred microliters of 0.5% HAc, 1 ml $CHCl_3$, and 800 μl 0.5% HAc were added. After each step, samples were vortexed for 30 s. The final mixture was centrifuged for 10 min at 1,892*g* at room temperature. After centrifugation, the lower layer of $CHCl_3$ was separated. This step was repeated two times by adding 1 ml $CHCl_3$. The separated $CHCl_3$ layers were combined and dried (N_2, 30°C). Samples were dissolved in 150 μl 25% $CHCl_3$/MeOH/H_2O/NH_3 (50/45/5/0.01 v/v/v/v) for further analysis.

High-performance Liquid Chromatography Tandem Mass Spectrometry

The relative concentrations of lysophosphatidylcholines and phosphatidylcholine species in supernatant of erythrocytes were determined using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). Ten microliters of extracted lipid sample was injected on the HPLC-MS/MS system. Chromatographic separation was achieved on a modular HPLC system (Surveyor; Thermo Finnigan, San Jose, CA) consisting of a cooled autosampler (T = 12°C), a low-flow quaternary MS pump, and an analytical HPLC column: LichroSpher Si60 (Merck, Darmstadt, Germany), 2 × 250 mm column, 5-μm particle diameter. Samples were eluted with a flow rate of 300 μl/min and a programmed linear gradient between solution B (chloroform–methanol, 97:3, v/v) and solution A (methanol–water, 85:15, v/v); A and B contained 1 ml and 0.1 ml 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient was: T = 0–10 min: 20% A to 100% A; T = 10–12 min, 100% A; T = 12–12.1 min: 100% A to 0% A; and T = 12.1–17 min, equilibration with 0% A. Total run-time, in-

cluding the equilibration, was 17 min. A splitter between the HPLC and MS was used for the introduction of the eluent in the MS by 75 μl/min.

MS/MS analyses were performed on a TSQ Quantum AM (Thermo Finnigan, Waltham, MA) operated in the positive ion electrospray ionization mode. The skimmer offset was set at 10 V; spray voltage was 3,600 V; and the capillary temperature was 300°C. In the optimized MS/MS experiments, argon was used as collision gas at a pressure of 0.07 Pa and a collision energy of 40 V. The parent ion scan of m/z 184.1 (m/z 400–1000, 1 s) was used for the quantization of the following precursor ions: m/z 468.3 (lysophosphatidylcholine 14:0, internal standard), m/z 496.3 (lysophosphatidylcholine 16:0), m/z 524.3 (lysophosphatidylcholine 18:0/platelet-activating factor [PAF] 16:0), m/z 522.4 (lysophosphatidylcholine 18:1), m/z 482.4 (LysoPAF 16:0), m/z 510.4 (LysoPAF 18:0), m/z 508.4 (LysoPAF 18:1), m/z 678.4 (phosphatidylcholine 28:0, I.S.), m/z 758.4 (phosphatidylcholine 34:2), and m/z 782.4 (phosphatidylcholine 36:2).

Storage-related Biochemical Changes in Human Erythrocytes

Healthy adult volunteers (n = 5) donated 1 unit of whole blood (500 ml), collected in citrate–phosphate–dextrose (70 ml), and stored for 12–18 h at 20–22°C. Leukoreduced erythrocytes were prepared by centrifugation for 8 min at 2,800*g*. After removal of plasma and buffy coat, 110 ml of the standard storage medium saline–adenine–glucose–mannitol was added *via* the filter to the packed red cells, which were subsequently leukoreduced by filtration. The erythrocytes were stored at 4°C according to National Blood Bank standards. Supernatants were collected at days 0, 35, and 42 and prepared by centrifugation for 10 min at 14,500*g* at 4°C to remove cells and acellular debris. Aliquots of supernatants were stored at −80°C for analysis of lysophosphatidylcholine and phosphatidylcholine levels.

Statistical Analyses

Data are expressed as mean ± SEM. A paired *t* test was used to compare the results of erythrocytes before and after storage. Comparisons between the rat groups were performed using Student *t* test, one-way ANOVA, followed by Dunnett's *post hoc* test. A *P* value less than 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 12.0 (SPSS, Chicago, IL) and Prism 4.0 (GraphPad Software, San Diego, CA).

Results

All animals completed the experimental protocol. Blood cultures from the blood products and from the rats collected at the end of the experimental protocol showed no outgrowth of bacteria.

Effect of Transfusion of Aged Rat Erythrocytes in Healthy Rats

Transfusion of aged erythrocytes resulted in endothelial neutrophil sequestration and edema in lung tissue (fig. 1), with a concomitant higher histopathology score compared with transfusion of fresh erythrocytes and saline control groups ($P < 0.05$, fig. 2). Aged erythrocytes also caused an increase of interleukin-6 and cytokine-induced neutrophil chemoattractant-3 concentrations in the lung homogenate of healthy animals ($P < 0.01$ compared with controls, fig. 3). Aged erythrocytes neither increase markers of pulmonary coagulation compared with fresh erythrocytes (TATc [mean ± SEM]: 1.5 ± 0.4 ng/ml *vs.* 1.6 ± 0.4 ng/ml, ns; fibrin degradation products: 94 ± 15.3 ng/ml *vs.* 99 ± 9.8 ng/ml, ns) nor impair fibrinolysis by reducing the activity of plasminogen activator (38% ± 7.4% *vs.* 36% ± 4.5%, ns) or increasing the activity of fibrinolytic inhibitor plasminogen activator inhibitor-1 (6.9 ± 1.9 *vs.* 6.4 ± 1.6 ng/ml, ns).

Effect of Transfusion of Aged Rat Erythrocytes in Lipopolysaccharide-primed Rats

To determine whether the "two-hit" effect could be reproduced in our syngeneic model, we repeated transfusion with aged erythrocytes in lipopolysaccharide-pretreated animals. In this experiment, lipopolysaccharide pretreatment resulted in lung injury, exemplified by neutrophil sequestration in the lung endothelium and pulmonary edema, with an elevated histopathology score ($P < 0.001$, figs. 1 and 2) and increased levels of interleukin-6 and cytokine-induced neutrophil chemoattractant-3 in lung homogenate ($P < 0.01$, fig. 3) compared with saline controls. Furthermore, lipopolysaccharide pretreatment increased pulmonary coagulation as shown by increased thrombin generation (as reflected by TATc, fig. 4) and increased fibrin degradation products levels. In addition, fibrinolysis was impaired, as evidenced by reduced the activity levels of the plasminogen activator (in percentage), caused by an increase in the levels of the fibrinolytic plasminogen activator inhibitor-1 compared with saline controls. Lipopolysaccharide pretreatment also increased TATc levels in plasma compared with saline controls, indicating increased systemic coagulation.

Transfusion of aged erythrocytes in lipopolysaccharide-primed animals did not further augment pulmonary inflammation, shown by an unaltered histopathology score (figs. 1 and 2) and a nonsignificant increase in pulmonary cytokine and chemokine levels compared with lipopolysaccharide-primed rats transfused with saline or fresh erythrocytes (fig. 3). Aged erythrocytes worsened pulmonary coagulopathy in lipopolysaccharide-primed animals, by increasing bronchoalveolar lavage fluid levels of TATc compared with lipopolysaccharide controls receiving saline or fresh erythrocytes ($P < 0.01$, fig. 4). Also, aged erythrocytes strongly contributed to impaired fibrinolysis in lipopolysaccharide-primed animals, decreasing the activity of plasminogen activator in the bronchoalveolar lavage fluid and increasing the level of plasminogen activator inhibitor-1 compared with lipopolysaccharide controls receiving fresh erythrocytes ($P < 0.05$ for

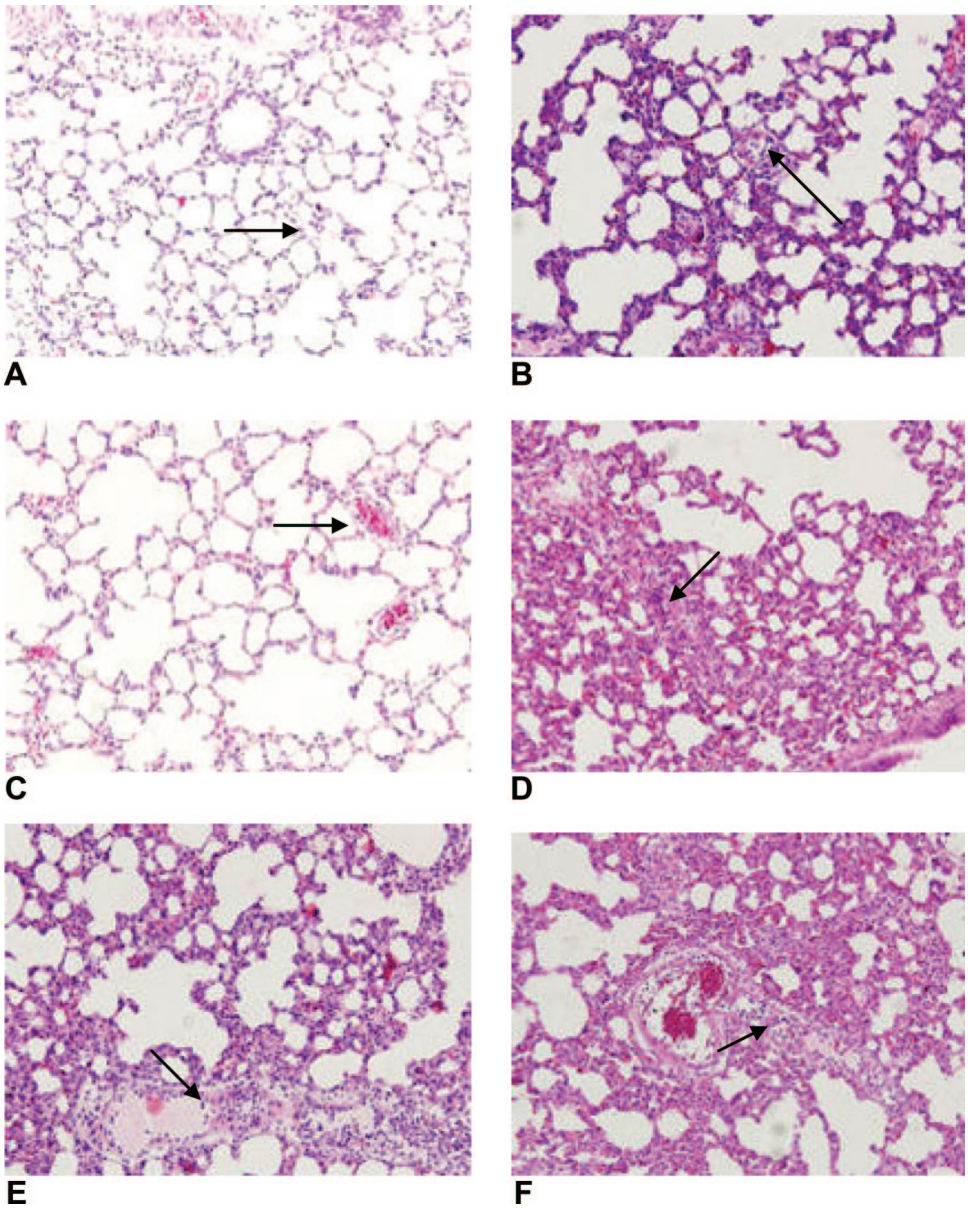

Fig. 1. Histologic sections of hematoxylin and eosin-stained rat lungs at 20× magnification. (*A*) Saline control, (*B*) lipopolysaccharide (LPS) control, (*C*) saline + erythrocytes day 0, (*D*) LPS + erythrocytes day 0, (*E*) saline + erythrocytes day 14, and (*F*) LPS + erythrocytes day 14. Normal vasculature (*arrow*, *A* and *C*), neutrophils sequestrated in the vasculature (*arrow*, *B*, *D–F*). N = 6 per group.

both). In addition, aged erythrocytes further augmented systemic coagulation, by increasing plasma TATc level compared with the lipopolysaccharide controls receiving fresh erythrocytes (fig. 5, $P < 0.001$).

Effect of Transfusion of Washed Aged Erythrocytes Versus *Supernatant of Aged Erythrocytes in Healthy and Lipopolysaccharide-primed Rats*

To determine whether lung injury was due to soluble factors in the storage medium or to the aged erythrocyte itself, aged erythrocytes were washed and separated from supernatant. Using these products, we repeated experiments in healthy and lipopolysaccharide-pretreated animals. In healthy rats, transfusion of both washed aged erythrocytes and supernatant of aged erythrocytes reproduced the findings of the previous experiment, increasing pulmonary cytokine and chemokine levels (data not shown). Washing of the aged erythrocytes did not prevent the onset of pulmonary inflammation in healthy rats.

In lipopolysaccharide-primed rats, transfusion of supernatant, but not of aged washed erythrocytes, worsened lung inflammation and coagulation, comparable with the previous experiment. Supernatant of aged erythrocytes increased pulmonary levels of interleukin-6 and cytokine-induced neutrophil chemoattractant-3 compared with rats receiving washed aged erythrocytes ($P < 0.01$, fig. 3), as well as an

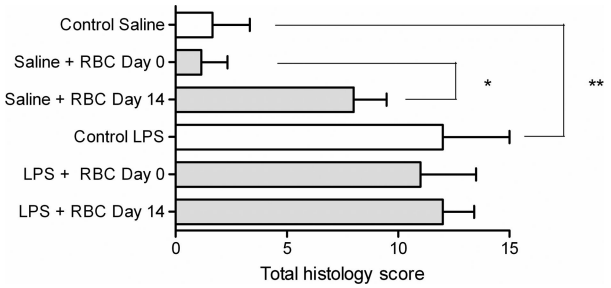

Fig. 2. Histology scores on lung injury. Lung injury score was significantly increased in lipopolysaccharide (LPS)-pretreated animals compared with saline controls. Aged erythrocytes (RBC day 14) increased lung injury compared with fresh erythrocytes (RBCs day 0) in healthy animals but did not further augment injury in LPS-pretreated animals. Data are presented as mean ± SEM * $P < 0.05$, ** $P < 0.001$. N = 6 per group. One-way analysis of variance followed by Dunnett's *post hoc* test.

increase in the levels of TATc and decrease in the activity of plasminogen activator in bronchoalveolar lavage fluid compared with those receiving washed aged erythrocytes ($P < 0.01$, fig. 4). Also, the increase in systemic levels of TATc caused by erythrocyte products was reproduced after transfusion of supernatant but not after transfusion of washed aged erythrocytes ($P < 0.001$, fig. 5).

Effects of Storage Time of Rat Erythrocytes on Biochemical Changes

To determine which factors in the supernatant are causative in inducing pulmonary injury, biochemical changes of erythrocyte products were analyzed. After 14 days of storage, erythrocytes had significant storage lesions, exemplified by an increase in potassium and lactate levels and a decrease in pH, sodium, and glucose compared with day 0 ($P < 0.01$ for all, table 1). Total hemoglobin concentration remained stable during storage, whereas hematocrit decreased during storage. Concentrations of lysophosphatidylcholine 16:0, lysophosphatidylcholine 18:0/PAF 16:0, LysoPAF 18:0, lysophosphatidylcholine 18:1, LysoPAF 16:0, and LysoPAF 18:1 did not increase during storage. In line with these results, the concentration of the biochemical precursors of lysophosphatidylcholines (phosphatidylcholines) remained stable. Interleukin-6 and tumor necrosis factor were not detectable in the supernatant of stored erythrocytes at either time point.

Effect of Storage Time of Human Erythrocytes on Lysophosphatidylcholine Accumulation

In contrast to our results, several previous studies found lysophosphatidylcholine accumulation in stored blood products.[15,17] Therefore, we performed additional studies using human erythrocytes. Comparable with the results with the

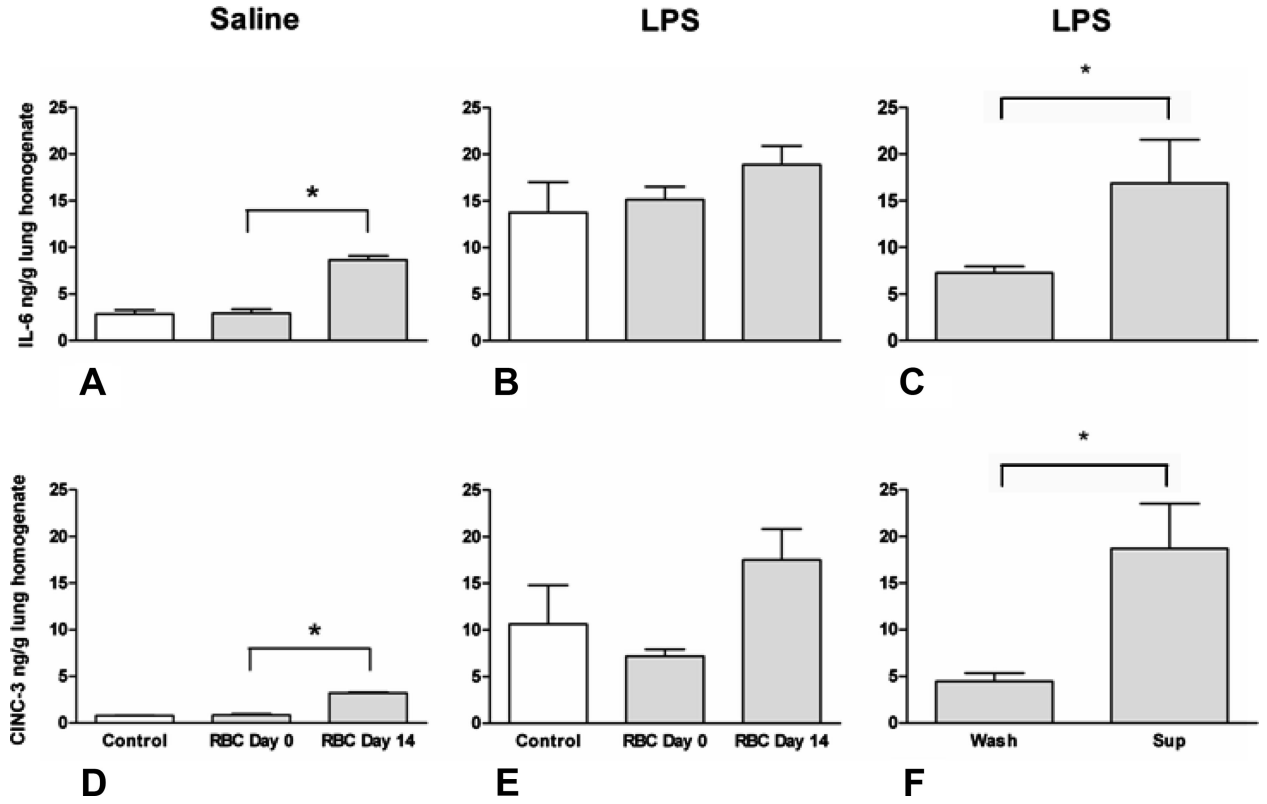

Fig. 3. Levels of interleukin (IL)-6 and cytokine-induced neutrophil chemoattractant (CINC)-3 in the lung homogenate of healthy animals (*A* and *D*) and LPS-primed animals infused with aged erythrocytes (RBCs day 14), fresh erythrocytes (RBCs, day 0), or saline (*B* and *E*; one-way analysis of variance followed by Dunnett's *post hoc* test). Additional experiments (*C* and *F*) show the effect of infusion of supernatant (Sup) of aged erythrocyte blood products or washed (Wash) aged erythrocytes in lipopolysaccharide (LPS)-primed animals (Student *t* test). Data are presented as mean ± SEM. * $P < 0.01$. N = 6 per group.

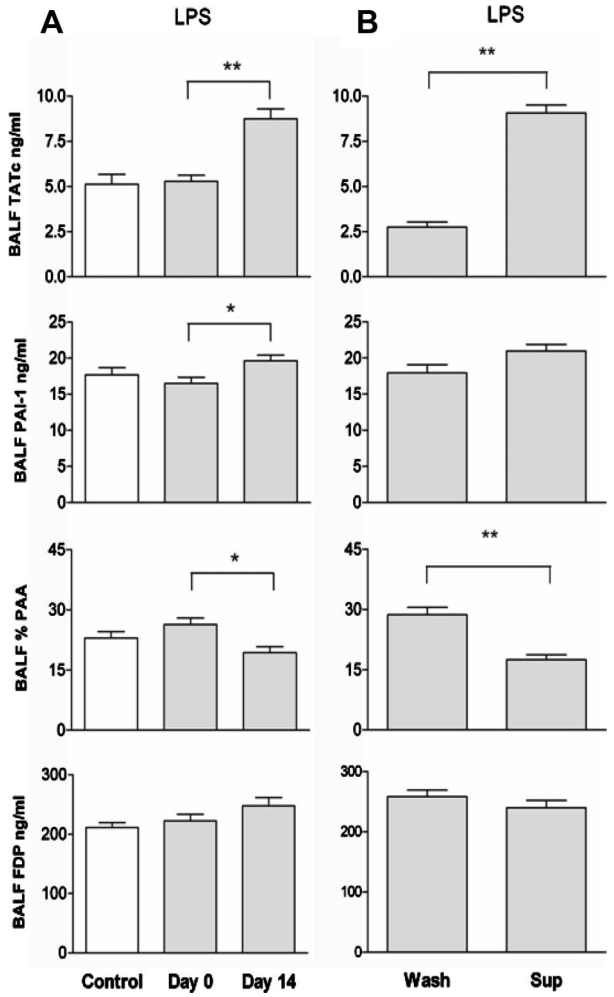

Fig. 4. Concentrations of thrombin-antithrombin complexes (TATc), plasminogen activator activity (PAA%), plasminogen activator inhibitor (PAI-1), and fibrin degradation products (FDP) in the bronchoalveolar lavage fluid (BALF) of lipopolysaccharide (LPS)-primed animals transfused with stored erythrocytes (day 14), fresh erythrocytes (day 0), saline (control), supernatant (Sup) of aged erythrocyte blood products, or washed (Wash) aged erythrocytes. Data are presented as mean ± SEM. Aged erythrocytes and supernatant of aged erythrocytes activate lung coagulation and decrease fibrinolysis as shown by an increase in TATc level in the BALF and decrease of PAA% and increase of PAI-1 levels in the BALF, respectively. * $P < 0.05$, ** $P < 0.01$. N = 6 per group. Student *t* test (*B*) and one-way analysis of variance followed by Dunnett's *post hoc* test (*A*).

rat erythrocyte products, concentrations of lysophosphatidylcholines did not increase in human erythrocyte products stored for 35 and even 42 days when compared with day 0 of storage (table 2). In line with these results, the concentration of the biochemical precursors of lysophosphatidylcholines (phosphatidylcholines) remained stable during storage.

Discussion

We describe a novel *in vivo* syngeneic rat erythrocyte transfusion model, using a clinical protocol for the preparation and storage of blood products according to National Blood Bank standards. In the model that we believe is clinically relevant, the main findings are as follows: (1) transfusion of aged erythrocytes resulted in mild lung inflammation in the absence of a priming "first hit," that is, in healthy lungs; (2) transfusion of aged erythrocytes increased lung injury in a "two-hit" TRALI model, which was characterized by profound pulmonary and systemic coagulopathy; and (3) lung injury in the "two-hit" model was abrogated by washing of the aged erythrocytes before transfusion.

Transfusion of aged erythrocytes induced lung inflammation in healthy lungs.[44] Notably, the amount of inflammation found was mild. This may partly be explained by a lack of immunogenicity. However, it can be speculated that the observed mild effects accumulate after repeated transfusions, which may contribute to respiratory complications. In accordance, observational clinical studies show that the number of erythrocytes transfused is associated with the onset of TRALI as well as with adverse outcome.[34,45] The clinical relevance of our findings remains to be determined in randomized trials investigating the effect of storage time of erythrocytes and onset of transfusion-related morbidity and mortality. Importantly, although the use of a syngeneic model does not reflect allogeneic blood transfusion, our model resembles the clinical situation more closely than the use of cross-species or an *ex vivo* design.[17,22,40] Using an in species transfusion model, we show that aged erythrocytes contribute to lung injury.

Previous models that have pointed toward a "two-hit" TRALI hypothesis are limited by *ex vivo* designs, use of blood products that were not manufactured according to clinical protocols or by the use of cross-species, including human blood products that were transfused in rat recipients.[17,22,40] In our syngeneic transfusion model, we confirm the "two-hit" TRALI hypothesis with the use of aged rat erythrocytes,[16,17,22] suggesting that effects of aged erythrocytes depend on priming status,[46,47] which is in line with the concept of the threshold model.[28] In this model, a threshold must be overcome to induce a TRALI reaction. Factors that determine the threshold are the clinical condition of the patient (*i.e.*, priming of the lung neutrophils) and the ability of the mediators in the transfusion to cause activation of primed neutrophils. Therefore, in the threshold model, severity of the TRALI reaction depends on both patient and transfusion-related factors. In accordance, we found that transfusion of aged erythrocytes induced mild lung inflammation in healthy rats, whereas lung injury increased when a priming hit preceded the transfusion. Of note, not all parameters of inflammation were augmented in the "two-hit" model. An explanation for this finding may be that inflammatory reactions, including extravasation of neutrophils, were already elicited by lipopolysaccharide priming, which could not be further enhanced by aged erythrocytes. However, results from our study underline the concept that critically ill patients with an inflammatory response may be susceptible to additional injury after a blood transfusion.[47,48] If indeed risk factors for acute lung injury of any origin predispose to

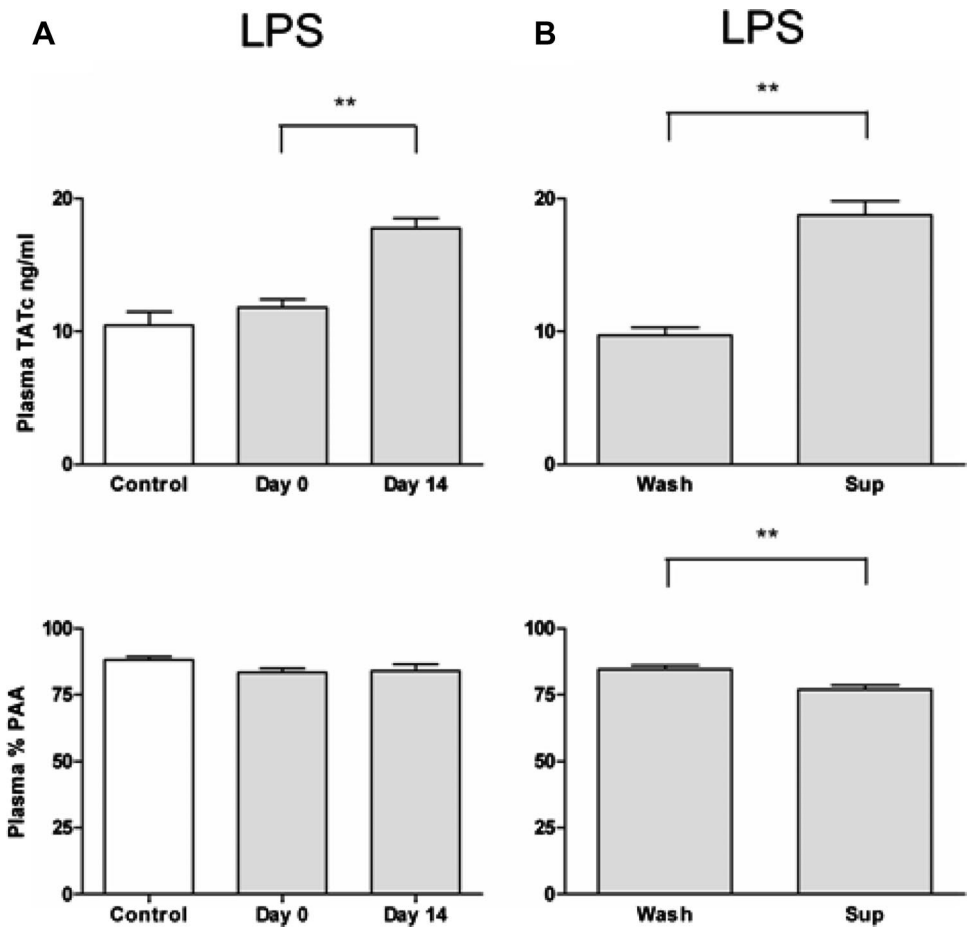

Fig. 5. Systemic concentrations of thrombin–antithrombin complexes (TATc) and plasminogen activator activity (PAA) in the plasma of lipopolysaccharide (LPS)-primed animals transfused with stored erythrocytes (day 14), fresh erythrocytes (day 0), saline (control), supernatant (Sup) of aged erythrocyte blood products, or washed (Wash) aged erythrocytes. Data are presented as mean ± SEM. Aged erythrocytes and supernatant of aged erythrocytes activate systemic coagulation and decrease fibrinolysis as shown by an increase in TATc level and decrease of PAA% in the plasma, respectively. ** $P < 0.01$. N = 6 per group. Student *t* test (*B*) and one-way analysis of variance followed by Dunnett's *post hoc* test (*A*).

TRALI, the multiple possible "first events" may explain the increased incidence of TRALI in the critically ill, when compared with the general hospital population.[29,49–51] Indeed, it is increasingly becoming clear that erythrocyte transfusion is associated with adverse outcome in patient groups that frequently suffer from inflammatory conditions, such as trauma and critically ill patients.[3] Our results underline the importance of restrictive transfusion protocols in these patient groups.

Our study extends previous findings, showing for the first time that aged erythrocytes cause increased coagulation and impaired fibrinolysis in the presence of primed neutrophils. erythrocytes are often considered passive bystanders in coagulation. However, it has long been known that aged erythrocytes have procoagulant activity,[52] which may result *via* increasing thrombin generation,[53,54] and activation of coagulation factors.[55] Our results suggest that in the presence of a "first hit," coagulopathy may be an important pathway in mediating lung injury after transfusion of aged erythrocytes. Of note, histopathologic examination of the lungs did not reveal evident thrombosis. This is in line with histopathologic findings in lungs of patients with acute lung injury due to other causes in which thrombi are not a frequent finding, even though coagulopathy is abundant.[56] Taken together, we suggest that lung injury induced by transfusion is comparable with the pathogenesis of acute lung injury/acute respiratory distress syndrome,[56,57] with regard to neutrophil extravasations and coagulopathy. Moreover, morbidity and mortality in critically ill patients developing TRALI may be comparable with patients with acute lung injury/acute respiratory distress syndrome, as evidenced by recent studies.[29,49,58] We suggest that TRALI should be regarded as a form of acute lung injury and not as a separate entity.

Proposed mechanisms of the induction of lung injury by storage of erythrocytes have included white blood cell-derived mediators, soluble factors in the supernatant, in particular lysophosphatidylcholines, or erythrocytes as the causative agents.[15,17,38] Our study suggests that the supernatant of the stored erythrocytes and not the aged erythrocyte itself caused inflammation in primed lungs. We found no increase in the levels of lysophosphatidylcholines or other proinflam-

Table 1. Storage-related Biochemical Changes in Rat Erythrocytes

	Whole Blood (Fresh)*	Erythrocytes Day 0†	Erythrocytes Day 14†
K^+ (mmol/l)	5.3 ± 0.2	3.6 ± 0.4	31.2 ± 2.1‡
Na^+(mmol/l)	154 ± 4.2	146 ± 1.8	126 ± 2.0§
pH	6.9 ± 0.1	7.1 ± 0.0	6.6 ± 0.01§
Lactate (mmol/l)	6.2 ± 1.2	7.0 ± 0.7	16.9 ± 3.1§
Glucose (mmol/l)	8.7 ± 0.5	18.5 ± 2.7	7.1 ± 0.3§
Leukocytes (×10/9/l)	5.4 ± 0.6	4.5 ± 0.8	3.6 ± 0.5
Hb (mmol/l)	6.0 ± 0.2	9.9 ± 0.3‖	9.7 ± 0.3
Hematocrit	0.34 ± 0.01	0.58 ± 0.02‖	0.53 ± 0.02§
LysoPC 16:0 (μM)		91.8 ± 6.9	87.3 ± 4.2
LysoPC 18:1 (μM)		22.9 ± 2.6	22.0 ± 2.4
LysoPC 18:0/ PAF 16:0 (μM)		2.9 ± 0.2	3.2 ± 0.1
LysoPAF 16:0 (μM)		2.6 ± 0.3	2.7 ± 0.1
LysoPAF 18:0 (μM)		2.9 ± 0.2	3.2 ± 0.1
LysoPAF 18:1 (μM)		1.2 ± 0.1	1.4 ± 0.2
PC 34:2 (μM)		42.6 ± 6.4	42.6 ± 5.3
PC 36:4 (μM)		16.2 ± 1.7	16.2 ± 0.8
TNF (pg/ml)		<62.5	<62.5
IL-6 (pg/ml)		<31.25	<31.25

Data are presented as mean ± SD.

*One-way analysis of variance, followed by Dunnett's *post hoc* test. † paired *t* test. ‡ $P < 0.001$, § $P < 0.01$, ‖ $P < 0.01$ erythrocytes day 14 compared with erythrocytes day 0 or erythrocytes day 0 compared with whole blood (fresh) (n = 5 batches).

Hb = hemoglobin; IL = interleukin; LysoPC = lysophosphatidylcholine; PAF = platelet-activating factor; PC = phosphatidylcholine; TNF = tumor necrosis factor.

Table 2. Storage-related Changes in Lysophophatidylcholines in Human Erythrocytes

	Erythrocytes Day 0	Erythrocytes Day 35	Erythrocytes Day 42
LysoPC 16:0 (μM)	13.4 ± 3.0	10.8 ± 4.4	10.6 ± 5.0
LysoPC 18:1 (μM)	3.2 ± 0.9	3.1 ± 1.1	2.7 ± 1.2
LysoPC 18:0/PAF 16:0 (μM)	0.8 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
LysoPAF 16:0 (μM)	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
LysoPAF 18:0 (μM)	0.3 ± 0.1	0.2 ± 0.2	0.2 ± 0.1
LysoPAF 18:1 (μM)	0.8 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
PC 34:2 (μM)	25.0 ± 6.5	25.0 ± 7.2	24.0 ± 7.9
PC 36:4 (μM)	9.8 ± 2.5	10.0 ± 3.0	9.3 ± 2.6

Data are presented as mean ± SD, n = 5 batches. One-way analysis of variance, followed by Dunnett's *post hoc* test, nonsignificant.

LysoPC = lysophosphatidylcholine; PAF = platelet-activating factor; PC = phosphatidylcholine.

matory factors after storage. As these findings are in contrast with previous studies,[15–18] we performed additional experiments with human erythrocyte blood products, which also did not show lysophosphatidylcholine accumulation. Blood product preparation for animal models varies considerably between laboratories, including use of storage solutions and leukoreduction, which may account for different study outcomes.[17,22,38,40,59–62] Also, clinical studies on the association between lysophosphatidylcholine concentration in transfused blood products and TRALI[63] show conflicting results.[18,29]

An alternative explanation for the mechanism of the detrimental effects in our model may be biochemical deterioration of the blood product. An increase in potassium and lactate levels and a decrease in pH in transfused products have been associated with morbidity and mortality in the pediatric patient.[64,65] Whether biochemical deterioration of blood products is able to induce coagulopathy is not answered by our study. Possibly, mediators produced by residual white blood cells may have contributed to lung injury. However, plasma from stored erythrocytes that were leukoreduced before storage were shown to induce lung injury in a two-event transfusion model,[22] rendering this hypothesis unlikely. Further research on the factor in the supernatant that elicits coagulopathy is required.

Of note, although results suggest that supernatant is the causative factor in primed lungs, both aged cells and supernatant elicit inflammation in healthy lungs. This interesting finding calls for further experiments with aged erythrocytes in various storage conditions. Also, it should be noted that the comparison of supernatant with aged erythrocytes in this study does not account for possible interactions between aged cells and aged supernatant. Furthermore, separating the products may have introduced other variables, such as a change in blood viscosity.

Our finding that washed aged erythrocytes inhibited lung injury in the "two-hit" model may have implications for the preparation and storage of erythrocytes. Washing of erythrocytes may reduce respiratory complications. Washing of stored blood without disturbing integrity of the aged erythrocyte seems a feasible procedure.[66,67] Alternatively, transfusion of fresh cells may only reduce pulmonary complications. A retrospective study suggested that cardiac surgery patients transfused with fresh erythrocytes (<14 days) compared with patients receiving aged erythrocytes (>14 days) had a reduced ventilation time and suffered less from respiratory insufficiency.[11] However, other clinical trials have not confirmed this finding.[68,69] Although it is clear that erythrocyte products deteriorate over time, a specific cutoff point in the risk-to-benefit ratio in transfusion related to the age of erythrocytes remains to be determined. However, our data suggest

that in certain patient populations (*e.g.*, the critically ill), washing of aged erythrocytes before transfusion or transfusion of fresh erythrocytes may only be a rational approach in reducing respiratory complications.

In conclusion, we show that transfusion of the supernatant of aged erythrocytes, but not washed aged erythrocytes, causes lung injury in a clinically relevant transfusion model, an effect that was modulated by the presence of a priming hit. In primed lungs, erythrocyte-induced lung injury was characterized by increased inflammation and coagulation and impaired fibrinolysis. The findings in the lipopolysaccharide-primed rats suggest that washing procedures of aged erythrocytes may decrease pulmonary complications after a blood transfusion. Given that critically ill and trauma patients are the patients who are most often transfused and that transfusion is the most common event preceding the development of acute lung injury[70–72] and an independent risk factor for acute lung injury,[32,36,45,73] efforts to reduce the adverse relation of blood transfusion and outcome are mandatory. Whether transfusion of fresh erythrocytes or washed aged erythrocytes reduces the increased risk for acquiring lung injury deserves further clinical studies.

The authors thank Richard Vlaar, B.S. (Technician, Sanquin Research, Department of Blood Cell Research, Amsterdam, The Netherlands), for assisting with the production of the rat blood components and Henk van Lenthe, Ph.D. (Technician, Genetic Metabolic Diseases, Academic Medical Center, Amsterdam, The Netherlands), for assisting with the lysophosphatidylcholine measurements.

References

1. Wells AW, Mounter PJ, Chapman CE, Stainsby D, Wallis JP: Where does blood go? Prospective observational study of red cell transfusion in north England. BMJ 2002; 325:803
2. Sullivan MT, Cotten R, Read EJ, Wallace EL: Blood collection and transfusion in the United States in 2001. Transfusion 2007; 47:385–94
3. Marik PE, Corwin HL: Efficacy of red blood cell transfusion in the critically ill: A systematic review of the literature. Crit Care Med 2008; 36:2667–74
4. Basran S, Frumento RJ, Cohen A, Lee S, Du Y, Nishanian E, Kaplan HS, Stafford-Smith M, Bennett-Guerrero E: The association between duration of storage of transfused red blood cells and morbidity and mortality after reoperative cardiac surgery. Anesth Analg 2006; 103:15–20
5. Keller ME, Jean R, LaMorte WW, Millham F, Hirsch E: Effects of age of transfused blood on length of stay in trauma patients: A preliminary report. J Trauma 2002; 53:1023–5
6. Leal-Noval SR, Jara-Lopez I, Garcia-Garmendia JL, Marin-Niebla A, Herruzo-Aviles A, Camacho-Larana P, Loscertales J: Influence of erythrocyte concentrate storage time on postsurgical morbidity in cardiac surgery patients. ANESTHESIOLOGY 2003; 98:815–22
7. Mynster T, Nielsen HJ: Storage time of transfused blood and disease recurrence after colorectal cancer surgery. Dis Colon Rectum 2001; 44:955–64
8. Offner PJ, Moore EE, Biffl WL, Johnson JL, Silliman CC: Increased rate of infection associated with transfusion of old blood after severe injury. Arch Surg 2002; 137:711–6
9. Purdy FR, Tweeddale MG, Merrick PM: Association of mortality with age of blood transfused in septic ICU patients. Can J Anaesth 1997; 44:1256–61
10. Zallen G, Offner PJ, Moore EE, Blackwell J, Ciesla DJ, Gabriel J, Denny C, Silliman CC: Age of transfused blood is an independent risk factor for postinjury multiple organ failure. Am J Surg 1999; 178:570–2
11. Koch CG, Li L, Sessler DI, Figueroa P, Hoeltge GA, Mihaljevic T, Blackstone EH: Duration of red-cell storage and complications after cardiac surgery. N Engl J Med 2008; 358:1229–39
12. Berezina TL, Zaets SB, Morgan C, Spillert CR, Kamiyama M, Spolarics Z, Deitch EA, Machiedo GW: Influence of storage on red blood cell rheological properties. J Surg Res 2002; 102:6–12
13. Ho J, Sibbald WJ, Chin-Yee IH: Effects of storage on efficacy of red cell transfusion: When is it not safe? Crit Care Med 2003; 31:S687–97
14. Silliman CC, Thurman GW, Ambruso DR: Stored blood components contain agents that prime the neutrophil NADPH oxidase through the platelet-activating-factor receptor. Vox Sang 1992; 63:133–6
15. Silliman CC, Clay KL, Thurman GW, Johnson CA, Ambruso DR: Partial characterization of lipids that develop during the routine storage of blood and prime the neutrophil NADPH oxidase. J Lab Clin Med 1994; 124:684–94
16. Silliman CC, Paterson AJ, Dickey WO, Stroneck DF, Popovsky MA, Caldwell SA, Ambruso DR: The association of biologically active lipids with the development of transfusion-related acute lung injury: A retrospective study. Transfusion 1997; 37:719–26
17. Silliman CC, Voelkel NF, Allard JD, Elzi DJ, Tuder RM, Johnson JL, Ambruso DR: Plasma and lipids from stored packed red blood cells cause acute lung injury in an animal model. J Clin Invest 1998; 101:1458–67
18. Silliman CC, Boshkov LK, Mehdizadehkashi Z, Elzi DJ, Dickey WO, Podlosky L, Clarke G, Ambruso DR: Transfusion-related acute lung injury: Epidemiology and a prospective analysis of etiologic factors. Blood 2003; 101:454–62
19. Silliman CC, Kelher M: The role of endothelial activation in the pathogenesis of transfusion-related acute lung injury. Transfusion 2005;45:109S–16S
20. Stack G, Snyder EL: Cytokine generation in stored platelet concentrates. Transfusion 1994; 34:20–5
21. Valeri CR, Collins FB: The physiologic effect of transfusing preserved red cells with low 2,3-diphosphoglycerate and high affinity for oxygen. Vox Sang 1971; 20:397–403
22. Kelher MR, Masuno T, Moore EE, Damle S, Meng X, Song Y, Liang X, Niedzinski J, Geier SS, Khan SY, Gamboni-Robertson F, Silliman CC: Plasma from stored packed red blood cells and MHC class I antibodies causes acute lung injury in a 2-event *in vivo* rat model. Blood 2009; 113:2079–87
23. Choi G, Vlaar AP, Schouten M, Van't Veer C, van der Poll T, Levi M, Schultz MJ: Natural anticoagulants limit lipopolysaccharide-induced pulmonary coagulation but not inflammation. Eur Respir J 2007; 30:423–8
24. Haitsma JJ, Schultz MJ, Hofstra JJ, Kuiper JW, Juco J, Vaschetto R, Levi M, Zhang H, Slutsky AS: Ventilator-induced coagulopathy in experimental *Streptococcus pneumoniae* pneumonia. Eur Respir J 2008; 32:1599–606
25. Hofstra JJ, Haitsma JJ, Juffermans NP, Levi M, Schultz MJ: The role of bronchoalveolar hemostasis in the pathogenesis of acute lung injury. Semin Thromb Hemost 2008; 34:475–84
26. Ware LB, Camerer E, Welty-Wolf K, Schultz MJ, Matthay MA: Bench to bedside: Targeting coagulation and fibrinolysis in acute lung injury. Am J Physiol Lung Cell Mol Physiol 2006; 291:L307–11
27. Mackie IJ, Bull HA: Normal haemostasis and its regulation. Blood Rev 1989; 3:237–50
28. Bux J, Sachs UJ: The pathogenesis of transfusion-related

acute lung injury (TRALI). Br J Haematol 2007; 136:788–99

29. Gajic O, Rana R, Winters JL, Yilmaz M, Mendez JL, Rickman OB, O'byrne MM, Evenson LK, Malinchoc M, Degoey SR, Afessa B, Hubmayr RD, Moore SB: Transfusion-related acute lung injury in the critically ill: Prospective nested case-control study. Am J Respir Crit Care Med 2007; 176: 886–91
30. Silliman CC: The two-event model of transfusion-related acute lung injury. Crit Care Med 2006; 34:S124–31
31. Corwin HL, Gettinger A, Pearl RG, Fink MP, Levy MM, Abraham E, MacIntyre NR, Shabot MM, Duh MS, Shapiro MJ: The CRIT Study: Anemia and blood transfusion in the critically ill—Current clinical practice in the United States. Crit Care Med 2004; 32:39–52
32. Croce MA, Tolley EA, Claridge JA, Fabian TC: Transfusions result in pulmonary morbidity and death after a moderate degree of injury. J Trauma 2005; 59:19–23
33. Engoren MC, Habib RH, Zacharias A, Schwann TA, Riordan CJ, Durham SJ: Effect of blood transfusion on long-term survival after cardiac operation. Ann Thorac Surg 2002; 74:1180–6
34. Gajic O, Rana R, Mendez JL, Rickman OB, Lymp JF, Hubmayr RD, Moore SB: Acute lung injury after blood transfusion in mechanically ventilated patients. Transfusion 2004; 44:1468–74
35. Hebert PC, Wells G, Blajchman MA, Marshall J, Martin C, Pagliarello G, Tweeddale M, Schweitzer I, Yetisir E: A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group. N Engl J Med 1999; 340:409–17
36. Silverboard H, Aisiku I, Martin GS, Adams M, Rozycki G, Moss M: The role of acute blood transfusion in the development of acute respiratory distress syndrome in patients with severe trauma. J Trauma 2005; 59:717–23
37. Vlaar AP, Binnekade JM, Schultz MJ, Juffermans NP, Koopman MM: Preventing TRALI: Ladies first, what follows? Crit Care Med 2008; 36:3283–4
38. Mangalmurti NS, Xiong Z, Hulver M, Ranganathan M, Liu XH, Oriss T, Fitzpatrick M, Rubin M, Triulzi D, Choi A, Lee JS: Loss of red cell chemokine scavenging promotes transfusion-related lung inflammation. Blood 2009; 113:1158–66
39. Vlaar AP, Zweers MM, Schultz MJ, Juffermans NP: Developing specific therapeutic strategies for transfusion-related acute lung injury. An overview of potentially useful animal models. Cardiovasc Hematol Agents Med Chem 2007; 5:319–26
40. Silliman CC, Bjornsen AJ, Wyman TH, Kelher M, Allard J, Bieber S, Voelkel NF: Plasma and lipids from stored platelets cause acute lung injury in an animal model. Transfusion 2003; 43:633–40
41. d'Almeida MS, Gray D, Martin C, Ellis CG, Chin-Yee IH: Effect of prophylactic transfusion of stored RBCs on oxygen reserve in response to acute isovolemic hemorrhage in a rodent model. Transfusion 2001; 41:950–6
42. Choi G, Hofstra JJ, Roelofs JJ, Florquin S, Bresser P, Levi M, van der Poll T, Schultz MJ: Recombinant human activated protein C inhibits local and systemic activation of coagulation without influencing inflammation during *Pseudomonas aeruginosa* pneumonia in rats. Crit Care Med 2007; 35:1362–8
43. Levi M, de Boer JP, Roem D, ten Cate JW, Hack CE: Plasminogen activation *in vivo* upon intravenous infusion of DDAVP. Quantitative assessment of plasmin-alpha 2-antiplasmin complex with a novel monoclonal antibody based radioimmunoassay. Thromb Haemost 1992; 67:111–6
44. Vlaar AP, de Korte D, Juffermans NP: The aged erythrocyte: Key player in cancer progression, but also in infectious and respiratory complications of blood transfusion? ANESTHESIOLOGY 2009; 111:444
45. Gong MN, Thompson BT, Williams P, Pothier L, Boyce PD, Christiani DC: Clinical predictors of and mortality in acute respiratory distress syndrome: Potential role of red cell transfusion. Crit Care Med 2005; 33:1191–8
46. Engelfriet CP, Reesink HW, Brand A, Palfi M, Popovsky MA, Martin-Vega C, Ribera A, Rouger P, Goldman M, Decary F, Freedman J, Lucas G, Navarette C, Neppert J, von Witzleben-Schurholz E, Lin M, Zupanska B: Transfusion-related acute lung injury (TRALI). Vox Sang 2001; 81: 269–83
47. Toy P, Popovsky MA, Abraham E, Ambruso DR, Holness LG, Kopko PM, McFarland JG, Nathens AB, Silliman CC, Stroncek D: Transfusion-related acute lung injury: Definition and review. Crit Care Med 2005; 33:721–6
48. Goldman M, Webert KE, Arnold DM, Freedman J, Hannon J, Blajchman MA: Proceedings of a consensus conference: Towards an understanding of TRALI. Transfus Med Rev 2005; 19:2–31
49. Rana R, Fernandez-Perez ER, Khan SA, Rana S, Winters JL, Lesnick TG, Moore SB, Gajic O: Transfusion-related acute lung injury and pulmonary edema in critically ill patients: A retrospective study. Transfusion 2006; 46:1478–83
50. Wallis JP, Lubenko A, Wells AW, Chapman CE: Single hospital experience of TRALI. Transfusion 2003; 43:1053–9
51. Wiersum-Osselton JC, Porcelijn L, van Stein D, Vlaar AP, Beckers EA, Schipperus MR: [Transfusion-related acute lung injury (TRALI) in the Netherlands in 2002–2005] Ned Tijdschr Geneeskd 2008; 152:1784–8
52. Quick AJ, Georgatsos JG, Hussey CV: The clotting activity of human erythrocytes: Theoretical and clinical implications. Am J Med Sci 1954; 228:207–13
53. Sweeney J, Kouttab N, Kurtis J: Stored red blood cell supernatant facilitates thrombin generation. Transfusion 2009; 49:1569–79
54. Horne MK III, Cullinane AM, Merryman PK, Hoddeson EK: The effect of red blood cells on thrombin generation. Br J Haematol 2006; 133:403–8
55. Iwata H, Kaibara M: Activation of factor IX by erythrocyte membranes causes intrinsic coagulation. Blood Coagul Fibrinolysis 2002; 13:489–96
56. Ware LB, Matthay MA: The acute respiratory distress syndrome. N Engl J Med 2000; 342:1334–49
57. Abraham E: Neutrophils and acute lung injury. Crit Care Med 2003; 31:S195–9
58. Vlaar AP, Binnekade J, Prins D, van Stein D, Schultz M, Juffermans N. Risk factors and outcome of transfusion related acute lung injury (TRALI) in the critically ill—A nested case control study. Crit Care Med 2010; 38:771–8
59. Atzil S, Arad M, Glasner A, Abiri N, Avraham R, Greenfeld K, Rosenne E, Beilin B, Ben-Eliyahu S: Blood transfusion promotes cancer progression: A critical role for aged erythrocytes. ANESTHESIOLOGY 2008; 109:989–97
60. Raat NJ, Verhoeven AJ, Mik EG, Gouwerok CW, Verhaar R, Goedhart PT, de Korte D, Ince C: The effect of storage time of human red cells on intestinal microcirculatory oxygenation in a rat isovolemic exchange model. Crit Care Med 2005; 33:39–45
61. Sachs UJ, Hattar K, Weissmann N, Bohle RM, Weiss T, Sibelius U, Bux J: Antibody-induced neutrophil activation as a trigger for transfusion-related acute lung injury in an ex vivo rat lung model. Blood 2006; 107:1217–9
62. Seeger W, Schneider U, Kreusler B, von WE, Walmrath D, Grimminger F, Neppert J: Reproduction of transfusion-related acute lung injury in an *ex vivo* lung model. Blood 1990; 76:1438–44
63. Nakazawa H, Ohnishi H, Okazaki H, Hashimoto S, Hotta H, Watanabe T, Ohkawa R, Yatomi Y, Nakajima K, Iwao Y, Takamoto S, Shimizu M, Iijima T: Impact of fresh-frozen

plasma from male-only donors *versus* mixed-sex donors on postoperative respiratory function in surgical patients: A prospective case-controlled study. Transfusion 2009; 49: 2434–41

64. Hall TL, Barnes A, Miller JR, Bethencourt DM, Nestor L: Neonatal mortality following transfusion of red cells with high plasma potassium levels. Transfusion 1993; 33:606–9
65. Ratcliffe JM, Elliott MJ, Wyse RK, Hunter S, Alberti KG: The metabolic load of stored blood. Implications for major transfusions in infants Arch Dis Child 1986; 61:1208–14
66. de Vroege R, Wildevuur WR, Muradin JA, Graves D, van OW: Washing of stored red blood cells by an autotransfusion device before transfusion. Vox Sang 2007; 92:130–5
67. Knichwitz G, Zahl M, Van AH, Semjonow A, Booke M: Intraoperative washing of long-stored packed red blood cells by using an autotransfusion device prevents hyperkalemia. Anesth Analg 2002; 95:324–5
68. van de Watering L, Lorinser J, Versteegh M, Westendord R, Brand A: Effects of storage time of red blood cell transfusions on the prognosis of coronary artery bypass graft patients. Transfusion 2006; 46:1712–8
69. Yap CH, Lau L, Krishnaswamy M, Gaskell M, Yii M: Age of transfused red cells and early outcomes after cardiac surgery. Ann Thorac Surg 2008; 86:554–9
70. Fowler AA, Hamman RF, Good JT, Benson KN, Baird M, Eberle DJ, Petty TL, Hyers TM: Adult respiratory distress syndrome: Risk with common predispositions. Ann Intern Med 1983; 98:593–7
71. Pepe PE, Potkin RT, Reus DH, Hudson LD, Carrico CJ: Clinical predictors of the adult respiratory distress syndrome. Am J Surg 1982; 144:124–30
72. Hudson LD, Milberg JA, Anardi D, Maunder RJ: Clinical risks for development of the acute respiratory distress syndrome. Am J Respir Crit Care Med 1995; 151:293–301
73. Chaiwat O, Lang JD, Vavilala MS, Wang J, MacKenzie EJ, Jurkovich GJ, Rivara FP: Early packed red blood cell transfusion and acute respiratory distress syndrome after trauma. ANESTHESIOLOGY 2009; 110:351–60

ANESTHESIOLOGY REFLECTIONS

"Olefiant Gas" Dollar-Bill Receipts

During the decades immediately before and after the American Civil War, many printers popularized advertising handbills and business receipts which resembled American paper money. Around 1858, a certain "D. Seller" paid $1.00 as a patent royalty "for personal use, the right to make and use the OLEFIANT GAS." Olefiant or "oil-making" referred to the alkene's ability, in the presence of chlorine, to form oily liquids (such as "Dutch oil," which was named after four Dutch chemists who produced it). Peak use of ethylene ("olefiant gas") as a general anesthetic in America would not occur until the mid-1920s to the mid-1930s. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in color in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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