HHS Public Access

Author manuscript

Ann Surg. Author manuscript; available in PMC 2020 March 10.

Published in final edited form as:

Ann Surg. 2017 January ; 265(1): 218–226. doi:10.1097/SLA.0000000000001648.

Acid Sphingomyelinase Inhibition in Stored Erythrocytes Reduces Transfusion-Associated Lung Inflammation

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Abstract

Objective: We aimed to identify the role of the enzyme acid sphingomyelinase in the aging of stored units of packed red blood cells (pRBCs) and subsequent lung inflammation after transfusion.

Summary Background Data: Large volume pRBC transfusions are associated with multiple adverse clinical sequelae, including lung inflammation. Microparticles are formed in stored pRBCs over time and have been shown to contribute to lung inflammation after transfusion.

Methods: Human and murine pRBCs were stored with or without amitriptyline, a functional inhibitor of acid sphingomyelinase, or obtained from acid sphingomyelinase-deficient mice, and lung inflammation was studied in mice receiving transfusions of pRBCs and microparticles isolated from these units.

Results: Acid sphingomyelinase activity in pRBCs was associated with the formation of ceramide and the release of microparticles. Treatment of pRBCs with amitriptyline inhibited acid sphingomyelinase activity, ceramide accumulation, and microparticle production during pRBC storage. Transfusion of aged pRBCs or microparticles isolated from aged blood into mice caused lung inflammation. This was attenuated after transfusion of pRBCs treated with amitriptyline or from acid sphingomyelinase-deficient mice.

Conclusions: Acid sphingomyelinase inhibition in stored pRBCs offers a novel mechanism for improving the quality of stored blood.

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A portion of this data was presented in abstract form at the 10th annual meeting of the Academic Surgical Congress, Las Vegas, NV, February 3, 2015.

Disclosure: The authors report no competing financial interests. Concepts and ideas herein are covered by a pending patent application: Serial No. 62/147,748. The author reports no conflicts of interest.

Keywords

acid sphingomyelinase; blood banking; ceramide; lung inflammation; microparticle

Hemorrhage and anemia are commonly encountered in the clinical setting, and current literature suggests the ideal treatment is transfusion of stored human packed red blood cells (pRBCs).[1,2] Previous studies suggest that pRBC transfusion is associated with worsened clinical outcomes (pneumonia, sepsis, and increased mortality) in specific patient populations, such as those with critical illness or undergoing cardiac surgery.[3–9] These effects are thought to be due to changes that occur in pRBCs as they age during storage.[3,10–13] Recent clinical evidence has disputed these negative outcomes[14–16]; however, "aged" pRBCs in these studies were only 20 to 30 days old and the results of these studies do not reflect current clinical practice.

Standard blood banking practice uses a first in, first out system whereby the oldest viable pRBCs are used first,[17] and current guidelines allow the transfusion of pRBCs up to 42 days of age.[3,18,19] As pRBCs age, they undergo biochemical and morpho-logical changes known as the erythrocyte "storage lesion."[17] A particularly harmful aspect of this storage lesion is the formation of microparticles. These subcellular particles range in size from 0.1 to 1.0 μm,[20–22] and have been shown to play a role in coagulation and complement factor activation,[23] neutrophil stimulation,[24] and lung inflammation.[24,25]

Sphingolipids, a class of lipids with a backbone of sphingoid bases, are essential cell membrane components, and play an important role in cell stress and apoptosis.[26] Previous studies implicate direct treatment of erythrocytes with sphingomyelinase in erythrocyte-derived microparticle formation in vitro.[27] Acid sphingomyelinase (ASM) activity has also been shown to correlate with microparticle concentrations in sickle cell patients.[28] To date, the role of sphingolipid metabolism in microparticle formation during PRBC storage is unknown. We hypothesized that the ASM plays a central role in microparticle formation during pRBC storage, and that genetic deletion or pharmacologic inhibition of ASM would reduce microparticle formation and mitigate the lung inflammation that occurs after transfusion of stored pRBCs.

METHODS

Animal Model

Male C57BL/6 mice aged 8 to 10 weeks were purchased from Jackson Laboratories. ASM-deficient mice ($ASM^{-/-}$) on a C57BL/6 background were used and compared with syngenic C57BL/6 wild-type mice as controls.[29] All experiments were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. Treatment animals received IV injections of blood components under isoflurane anesthesia, whereas sham animals under-went isoflurane anesthesia alone.

Blood Banking and Treatment

Human pRBCs were purchased from Hoxworth Blood Bank (Cincinnati, OH) and stored at 4°C for 42 days according to standard blood banking practice.[30] These units were leukoreduced before storage as a matter of routine. Murine pRBCs were prepared and stored as previously described,[24] and then stored in Eppendorf tubes as 1 mL “units” at 4°C for 14 days. Fresh pRBCs were prepared and used within 8 hours of preparation. These murine pRBCs were not routinely leukoreduced before storage. Where used, amitriptyline and fluoxetine (Sigma-Aldrich, St Louis, MO) were dissolved in normal saline (0.9% sodium chloride) and added to mouse and human pRBCs in a 1:10 dilution before storage. Except for dose–response experiments, amitriptyline treatment refers to a 125 μM concentration of amitriptyline in pRBCs.

Blood Component Isolation

Erythrocytes were pelleted from pRBC samples by centrifugation at 2000g for 10 minutes at 4°C. The supernatant from this spin was then centrifuged at 10,000g for 10 minutes at 4°C to pellet any remaining cells or platelets. The microparticle-rich supernatant from this second spin was used to characterize microparticle concentrations. To remove cytokines, residual amitriptyline, or soluble mediators in the serum for microparticle add-back experiments, microparticles were pelleted using a 20,000g spin for 30 minutes at 4°C and resuspended in 200 μL 0.9% sodium chloride for injections.[31]

ASM Activity

ASM activity in erythrocyte membranes and microparticle-rich supernatants was measured as previously described.[29] Membrane fragments were prepared by repeated osmotic and mechanical lysis of erythrocytes. Samples were shock-frozen, diluted in 250 mM sodium acetate (pH 5.0), 0.1% NP40, and incubated with [^{14}C]-sphingomyelin (Perkin Elmer, specific activity = 2 GBq/mmol) for 30 minutes at 37°C. Before use the substrate was dried, resuspended in 250 mM sodium acetate (pH 5.0), 0.1% NP40, and sonicated for 10 minutes in a bath sonicator (Branson) to obtain micelles. The enzyme reaction was terminated by extraction in 1 mL $CHCl_3$/CH_3OH (2:1, v/v). Samples were vortexed for 15 seconds and centrifuged at 14,000 rpm for 5 minutes. An aliquot of the aqueous phase was applied for liquid scintillation counting. Hydrolysis of [^{14}C]-sphingomyelin by ASM results in release of [^{14}C]-choline chloride into the aqueous phase, whereas ceramide and unreacted [^{14}C]-sphingomyelin remain in the organic phase. Therefore, the release of [^{14}C]-choline chloride serves to determine the activity of ASM. Results were normalized to protein concentration.

Ceramide Measurement

Ceramides were extracted and quantified as recently described.[32] Briefly, lipid extraction was performed using C17-ceramide as internal standard. Sample analysis was carried out by rapid-resolution liquid chromatography-MS/MS using a Q-TOF 6530 mass spectrometer (Agilent Technologies, Waldbronn, Germany) operating in the positive ESI mode. The precursor ions of ceramides [C16-ceramide (m/z 520.508), C17-ceramide (m/z 534.524), C18-ceramide (m/z 548.540), C20-ceramide (m/z 576.571), C22-ceramide (m/z 604.602), C24-ceramide (m/z 632.634), C24:1-ceramide (m/z 630.618)] were cleaved into the

fragment ion of m/z 264.270. Quantification was performed with Mass Hunter Software (Agilent Technologies).

Ceramide was also measured using a DAG kinase assay as previously described.[29] Lipids were extracted in $CHCl_3/CH_3OH$/1 NHCl (100:100:1, v/v/v), the organic phase was collected and dried, and lipids were solubilized in 20 μL 7.5% N-octyl glucopyranoside, 5 mM cardiolipin, 1 mM DETAPAC, and sonicated in a bath sonicator for 10 minutes. Fifty microliter of DAG kinase reaction buffer (100 mM imidazole/HCl [pH 6.6], 100 mM NaCl, 25 mM $MgCl_2$, 2 mM EDTA, 2.8 mM DTT, 5 μM ATP, 10 μCi [^{32}P]-γATP) and 0.01 U DAG kinase (BML-SE100; Enzo Life Sciences, East Farmingdale, NY) in 10 μL of 1 mM DETAPAC (pH 6.6) and 10 mM imidazole were added. The kinase reaction was performed for 30 minutes at room temperature.

Samples were then re-extracted in 1 mL $CHCl_3/CH_3OH$/1 NHCl (100:100:1, v/v/v), 170 μL buffered salt solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES [pH 7.2]) were added, followed by addition of 30 μL of 100 mM EDTA solution per sample. Samples were vortexed, the organic phase removed, and dried lipids were dissolved in 20 μL $CHCl_3/CH_3OH$ (2:1, v/v) per sample and then separated on a Silica G60 thin layer chromatography plate (Merck, Darmstadt, Germany) with a solvent system consisting of $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (10:4:3:2:1, v/v/v/v/v). The plate was analyzed using a Fuji phosphoimager. Ceramide spots were identified by co-migration with a C_{16}/C_{24}-ceramide standard and converted to ceramide amounts using a standard curve. Results were normalized to protein concentration.

Microparticle Quantification

To determine the concentration of microparticles in pRBCs, microparticle-rich supernatants were diluted in RPMI-1640 medium (Life Technologies, Carlsbad, CA), and Nanoparticle Tracking Analysis (Nanosight, Malvern Instruments, Malvern, UK) was used to identify particles between 50 and 1000 nm. For murine pRBCs, only particles that stained positively for glycophorin A (ter-119; BD Biosciences, San Jose, CA) were quantified. Owing to significant interunit variability in microparticle counts when analyzing human erythrocytes, microparticle concentrations are represented as fold changes from baseline for each unit.

Quantification of microparticles by flow cytometry, although the most common technique, is limited due to the small size of these particles.[20] Microparticle studies using nanoparticle tracking analysis and dynamic light scattering have determined the average size of microparticles to range from 100 to 300 nm.[33,34] However, the lower limit of detection by flow cytometry is 488 nm.[35] Therefore, much of the existing literature characterizing these microparticles can only be interpreted as a description of microparticles more than 500 nm, which misses the majority of microparticles. For this reason, we used nanoparticle tracking analysis to quantify microparticle formation in stored pRBCs, and found that amitriptyline treatment reduced the formation of microparticles of all sizes.

Measurement of Lung Inflammation

To study the effect of amitriptyline treatment on lung inflammation after blood transfusion, mice received 200 μL of pRBCs or microparticles under inhaled isoflurane anesthesia.

Lungs were taken 6 hours after transfusion for histologic evaluation. Histologic lung sections were examined for lung inflammation with hematoxylin & eosin (H&E) and for leukocyte infiltration with anti-Gr-1 antibodies (BD Biosciences, Franklin Lakes, NJ). Scoring and quantification were done by a member of the research team blinded to the treatment groups.

Lung inflammation scores were calculated as described previously.[36,37] In brief, 10 representative low-power (100×) images were taken of H&E-stained lung sections. Areas of thickened or aberrant lung architecture were calculated as a percentage of total lung area, and each image was given a score: 0 points—0%, 1 point—0% to 25%, 2 points—25% to 50%, 3 points—50% to 100%. The anti-Gr-1 antibody used (clone RB6–8C5) preferentially stains granulocytes with mild cross-reactivity for monocytes; thus, differences in Gr-1 cell counts likely represent neutrophil recruitment to the lungs.[38] For leukocyte quantification, 10 representative high-power (400×) images were taken of anti-Gr-1 stained lung sections, positively stained cells were quantified in each image. Average scores/counts were determined for each lung. Values are reported as means with standard error.

RESULTS

Aged Blood Transfusion Results in Lung Inflammation

In initial experiments, we examined the effect of transfusion of fresh and aged pRBCs on lung inflammation in mice. Lung specimens from mice transfused with aged pRBCs demonstrated a heterogeneous inflammatory pattern with areas of marked hemorrhage, tissue thickening, and abnormal architecture (Fig. 1A–D), and increased numbers of Gr-1 positive cells in the lungs (Fig. 1E–H). These findings were not present in mice that received fresh pRBCs (Fig. 1A–H), and indicate that transfusion of stored pRBCs results in increased lung inflammation and leukocyte infiltration in the lung.

ASM Activity Drives Microparticle Formation in Stored pRBCs

Previous studies suggest that microparticles accumulate in pRBCs during storage.[21,22] The current FDA-approved shelf life for human pRBCs is 42 days,[30] which we have previously shown to correspond to 14 days of storage for murine pRBCs.[39] Quantification of microparticle concentrations during standard storage conditions demonstrated that microparticles increase during storage of human (Fig. 2A) and murine (Fig. 2B) pRBCs.

To investigate the role of ASM in microparticle formation during pRBC storage, we stored erythrocytes in the presence of amitriptyline, a known functional inhibitor of ASM.[40] Amitryptiline inhibits ASM by displacing the enzyme from lysosomal or plasma membranes, resulting in the degradation of the enzyme in lysosomes or the release of the enzyme from the cell surface. Thus, relatively high concentrations of amitriptyline were used to displace ASM in the high number of erythrocytes present in blood samples. Nanoparticle tracking analysis revealed that amitriptyline treatment resulted in a dose-dependent reduction of microparticle formation in both human and murine pRBCs during storage (Fig. 2C, D). To determine if this finding was specifically related to ASM inhibition, we repeated this experiment in murine pRBCs with fluoxetine, another functional inhibitor

of ASM.[40] Fluoxetine also decreased microparticle formation in pRBCs during storage (Fig. 2E), suggesting that these findings are caused by functional inhibition of ASM rather than a nonspecific action of either drug. High concentrations of amitriptyline (>250 μM) were associated with increasing hemolysis (unpublished observations), although microparticle reduction was observed in mouse experiments starting at much lower doses and most significant at 125 μM amitriptyline. Thus, we used 125 μM amitriptyline as the treatment concentration for subsequent in vivo experiments.

To investigate the potential mechanism by which amitriptyline reduced microparticle formation in stored pRBCs, we assayed these units for ASM activity and ceramide concentration during storage. ASM activity on aged murine microparticles was increased compared with fresh (Fig. 3A), whereas ASM activity was decreased on stored erythrocytes as compared with fresh (Fig. 3C), suggesting that ASM changes location and is concentrated in extracellular microparticles over the course of pRBC storage. Amitriptyline reduced ASM activity on both microparticles and erythrocytes from aged murine pRBCs. Subsequently, ceramide concentrations were also reduced in microparticles (Fig. 3B) and erythrocytes (Fig. 3D, E) from aged murine pRBCs, most notably C16 and C18. Analysis of ASM and ceramide from microparticles isolated from stored human and murine pRBCs revealed the same dose-dependent reduction with amitriptyline treatment (Fig. 3F, G). These data provide evidence that addition of amitriptyline at the time of pRBC preparation reduces ASM activity during storage, which is associated with decreased ceramide concentrations.

Inhibition of ASM on Microparticles Reduces Transfusion-associated Lung Inflammation

To investigate the effect of ASM inhibition in pRBC units on subsequent lung inflammation after transfusion, we transfused mice with pRBCs that had been stored with or without amitriptyline. Mice receiving transfusions of pRBCs treated with amitriptyline at the time of storage demonstrated significantly less lung inflammation than those receiving pRBCs treated with vehicle alone (Fig. 4). Transfusion with vehicle-treated pRBCs was associated with marked alveolar thickening, distortion of normal lung architecture, and microvascular congestion. These effects were greatly reduced by storage of pRBCs with amitriptyline (Fig. 4A–C). Leukocyte recruitment to the lungs was also significantly reduced in mice that received amitriptyline-treated pRBCs as compared with those receiving pRBCs treated with vehicle only (Fig. 4D–F).

Prior data from our laboratory suggest that microparticles from stored pRBCs play a key role in lung inflammation after transfusion.[24] To investigate the relationship between amitriptyline treatment and microparticle-induced lung inflammation, we isolated microparticles from equal volumes of pRBCs stored with or without amitriptyline and resuspended them in normal saline for injection. Mice given microparticles isolated from vehicle-treated pRBCs demonstrated lung inflammation similar to those receiving untreated pRBCs (Fig. 5A). Lung inflammation was sharply attenuated in mice receiving microparticles isolated from amitriptyline-treated pRBCs (Fig. 5A–C). Staining for Gr-1 of these specimens indicated that microparticles isolated from pRBCs stored with amitriptyline induced less pulmonary leukocyte infiltration than those stored with vehicle (Fig. 5 D–F). This indicates that the reduced concentration of microparticles in amitriptyline-treated

pRBCs leads to decreased lung inflammation in a mouse model. Next, to investigate changes in microparticle quality, we isolated and injected equal numbers of microparticles from pRBCs stored with or without amitriptyline. The microparticles were again pelleted before injection to remove any soluble factors. Mice treated with microparticles isolated from amitriptyline-treated pRBCs demonstrated reduced lung inflammation (Fig. 6). Taken together, these experiments demonstrate that ASM-related changes in microparticle number and quality each contribute to lung inflammation after pRBC transfusion.

Aged pRBCs from ASM-deficient Mice Do Not Cause Lung Inflammation

Finally, we investigated the effect of transfusing pRBCs acquired from ASM-deficient mice. pRBCs were prepared from wild-type or ASM-deficient mice, stored under standard storage conditions, and then transfused into wild-type mice. Stored ASM-deficient pRBCs caused substantially less lung inflammation compared with wild-type pRBCs (Fig. 7A–C). We also found a similar reduction in leukocyte recruitment to the lungs after transfusion with ASM-deficient pRBCs (Fig. 7D–F). These findings mirrored the results from transfusion of pRBCs treated with amitriptyline, proving that the ASM/ceramide system plays a central role in the detrimental effect of aged pRBCs on lung inflammation.

DISCUSSION

In the present study, we provide new evidence regarding the role of ASM in the aging of stored erythrocytes. We have shown that treatment of murine and human pRBCs with amitriptyline resulted in a dose-dependent reduction in ASM activity, ceramide concentration, and microparticle formation during storage. Transfusion of pRBCs treated with amitriptyline or deficient in ASM led to less lung inflammation in mice when compared with wild-type pRBCs treated with vehicle alone. Transfusion of microparticles from these stored pRBCs yielded similar results, suggesting that microparticles play a key role in lung inflammation after transfusion of stored pRBCs. Thus, genetic or pharmacologic inhibition of ASM in stored pRBCs abrogates lung inflammation after transfusion of aged blood. This offers a potential opportunity to improve the quality of stored erythrocytes used for human blood transfusion.

We have also provided novel insight regarding the role of sphingolipids in microparticle production during pRBC storage. Prior research has implicated multiple causes of microparticle production under these conditions, including ATP depletion[41] and aggregation of band 3 protein.[42] It is likely that the decrease in pH and increase in metabolic stress during pRBC storage contribute to ASM activation and subsequent microparticle production. Our studies show that an inhibition of the ASM reduces the production of microparticles, even if the pH in blood samples is not pH 5.0, the optimal pH for ASM activity. However, the lipid composition of the membrane environment determines the affinity of the ASM and allows the enzyme to be active at higher pH.[43]

Our findings indicate that ASM inhibition leads to a change in both microparticle quantity and quality that ultimately prevents lung inflammation. Less lung inflammation was observed after injecting microparticles isolated from equal volumes of amitriptyline-treated pRBCs, indicating that microparticle quantity affects lung inflammation. However, when we

injected equal numbers of microparticles from untreated or amitriptyline-treated pRBCs, the pretreatment with amitriptyline also reduced lung inflammation. Because we injected purified microparticles into the recipient mice, the effect of amitriptyline is very likely mediated by a direct effect on these microparticles, that is the reduction of ASM content and/or the ceramide concentration. It is possible that ASM in microparticles directly acts on endothelial cells in the lung or is released from microparticles to bind on endothelial cells. The activity of microparticle-derived ASM on endothelial cells may trigger endothelial changes, for instance, of tight junctions[29] that lead to inflammation. This notion is consistent with observations that ASM activity has been associated with multiple inflammatory states.[44] If the microparticles fuse with endothelial cells, ceramide may have similar effects on the integrity of endothelial cells in the lung and may affect integrity of tight junctions,[29] and may even induced endothelial cell apoptosis.[45]

There is ongoing debate regarding the relationship between pRBC storage time and adverse effects after transfusion. Previous research suggests that the use of aged pRBCs leads to adverse patient outcomes.[11,12] However, recent randomized trials have suggested that there is no difference between fresh and aged pRBCs.[14–16] Of note, these studies considered pRBCs “fresh” if they are less than 6 days old and “old” if they are greater than 20 days old. We have shown that changes associated with pRBC aging, specifically microparticle production, increase dramatically toward the end of their 42-day shelf life. Thus, pRBCs that are 10, 20, or even 30 days old have similar characteristics, and only pRBCs near the end of their shelf life may be harmful to patients. In our opinion, all pRBCs should be used as early as possible after donation to avoid the potential adverse effects of transfusing older pRBCs. This practice has been pursued in some circumstances, with current US military clinical practice guidelines recommending use of fresh whole blood for casualties in whom a massive transfusion is anticipated, but has not been widely implemented, especially in civilian practice.[46] If it proves feasible in the clinical setting, ASM inhibition in pRBCs at the time of storage may improve the quality of stored pRBCs in areas where ample supply of human blood products is scarce and pRBCs are routinely used at the end of their shelf life.

One concern regarding the potential use of ASM inhibition in pRBC storage is the dose of amitriptyline that could be delivered to the transfusion recipient. Previously reported toxic doses of amitriptyline in animals range from 15 to 30 mg/kg.[47,48] If amitriptyline levels remained unchanged during pRBC storage, which is unlikely, this means that a 70 kg patient could demonstrate toxicity starting at doses near 1 g. To receive this dose, a patient would need to be transfused 25.5 L (or >100 units) of pRBCs with an amitriptyline concentration of 125 μM. It is unlikely that toxicity would be achieved under these circumstances as the patient would also be actively bleeding and thus losing amitriptyline at the same time. In summary, toxic effects due to the direct delivery of amitriptyline are unlikely, but continued work in our laboratory investigating the pharmacokinetics of amitriptyline in stored pRBCs.

One limitation of our study is the absence of a severe “lung injury” in our blood transfusion model. However, it should be noted that this represents a low volume transfusion in an otherwise healthy animal and not a clinically defined massive pRBC transfusion.[1] Mice received blood transfusions of 200 μL, which represents a transfusion equivalent to approximately 10% of the circulating blood volume. A similar transfusion in a typical adult

patient, with a 5 L circulating blood volume, would be 500 mL, or approximately 2 units of standard pRBCs. In this setting, we reliably observed histopathological evidence of lung inflammation in mice receiving both aged pRBCs and microparticles isolated from these units, and this lung inflammation was not evident in mice receiving amitriptyline-treated pRBCs. Indeed, this benefit would likely be enhanced in mice, and human patients, receiving large volumes of pRBC transfusion in the setting of anemia or hemorrhagic shock. Another potential limitation of this study is that our murine pRBCs were not leukoreduced, a practice used by many countries.[49] Previous studies indicate that the large majority of microparticles generated during pRBC storage are erythrocyte-derived, and this ratio is not significantly altered with leukoreduction.[50] Moreover, both human (leukoreduced) and murine (nonleukoreduced) pRBCs in this study demonstrated similar effects with ASM inhibition.

In summary, our findings provide novel evidence that sphingolipid metabolism plays a crucial role in the aging of stored pRBCs. ASM activity during pRBC storage is associated with microparticle production, and these microparticles contribute to lung inflammation after pRBC transfusion. Moreover, our data demonstrate that amitriptyline prevents microparticle production and improves the quality of aging pRBCs. This research is promising as a method to extend the shelf-life of aged blood and ameliorate aspects of the adverse effects of blood transfusion.

ACKNOWLEDGMENTS

The authors thank Rebecca Schuster for expert technical assistance with histological sections and Lou Ann Friend for assistance with in vivo models.

Funding: This work was supported in part by grant R01 GM107625 from the National Institute of General Medical Sciences of the US National Institutes of Health and DFG grant GU 335/30–1.

REFERENCES

1. Holcomb JB, del Junco DJ, Fox EE, et al. The prospective, observational, multicenter, major trauma transfusion (PROMMTT) study: comparative effectiveness of a time-varying treatment with competing risks. JAMA Surg. 2013;148:127–136. [PubMed: 23560283]
2. Holcomb JB, Donathan DP, Cotton BA, et al. Prehospital transfusion of plasma and red blood cells in trauma patients. Prehosp Emerg Care. 2015;19:1–9. [PubMed: 24932734]
3. Weinberg JA, McGwin G, Marques MB, et al. Transfusions in the less severely injured: does age of transfused blood affect outcomes? J Trauma. 2008;65: 794–798. [PubMed: 18849793]
4. Purdy FR, Tweeddale MG, Merrick PM. Association of mortality with age of blood transfused in septic ICU patients. Can J Anaesth. 1997;44:1256–1261. [PubMed: 9429042]
5. Zallen G, Offner PJ, Moore EE, et al. Age of transfused blood is an independent risk factor for postinjury multiple organ failure. Am J Surg. 1999;178:570–572. [PubMed: 10670874]
6. Offner PJ, Moore EE, Biffl WL, et al. Increased rate of infection associated with transfusion of old blood after severe injury. Arch Surg. 2002;137: 711–716. [PubMed: 12049543]
7. Koch CG, Li L, Sessler DI, et al. Duration of red-cell storage and complications after cardiac surgery. N Engl J Med. 2008;358:1229–1239. [PubMed: 18354101]
8. Horvath KA, Acker MA, Chang H, et al. Blood transfusion and infection after cardiac surgery. Ann Thorac Surg. 2013;95:2194–2201. [PubMed: 23647857]
9. Spinella PC, Carroll CL, Staff I, et al. Duration of red blood cell storage is associated with increased incidence of deep vein thrombosis and in hospital mortality in patients with traumatic injuries. Crit Care. 2009;13:R151. [PubMed: 19772604]

10. Mangalmurti NS, Xiong Z, Hulver M, et al. Loss of red cell chemokine scavenging promotes transfusion-related lung inflammation. Blood. 2009;113: 1158–1166. [PubMed: 19064726]
11. Weinberg JA, McGwin G, Vandromme MJ, et al. Duration of red cell storage influences mortality after trauma. J Trauma. 2010;69:1427–1431. [PubMed: 21150522]
12. Tan H, Bi J, Wang Y, et al. Transfusion of old RBCs induces neuroinflammation and cognitive impairment. Crit Care Med. 2015;43:e276–e286. [PubMed: 25860202]
13. Sauaia A, Moore FA, Moore EE, et al. Early predictors of postinjury multiple organ failure. Arch Surg. 1994;129:39–45. [PubMed: 8279939]
14. Lacroix J, Hébert PC, Fergusson DA, et al. Age of transfused blood in critically ill adults. N Engl J Med. 2015;372:1410–1418. [PubMed: 25853745]
15. Steiner ME, Ness PM, Assmann SF, et al. Effects of red-cell storage duration on patients undergoing cardiac surgery. N Engl J Med. 2015;372:1419–1429. [PubMed: 25853746]
16. Walsh TS, McArdle F, McLellan SA, et al. Does the storage time of transfused red blood cells influence regional or global indexes of tissue oxygenation in anemic critically ill patients? Crit Care Med. 2004;32:364–371. [PubMed: 14758149]
17. Koch CG, Figueroa PI, Li L, et al. Red blood cell storage: how long is too long? Ann Thorac Surg. 2013;96:1894–1899. [PubMed: 24090578]
18. Brown CH, Grega M, Selnes OA, et al. Length of red cell unit storage and risk for delirium after cardiac surgery. Anesth Analg. 2014;119:242–250. [PubMed: 24859077]
19. Shapiro MJ, Gettinger A, Corwin HL, et al. Anemia and blood transfusion in trauma patients admitted to the intensive care unit. J Trauma. 2003;55: 269–273. [PubMed: 12913636]
20. Lacroix R, Robert S, Poncelet P, et al. Overcoming limitations of microparticle measurement by flow cytometry. Semin Thromb Hemost. 2010;36:807–818. [PubMed: 21049381]
21. Xiong Z, Cavaretta J, Qu L, et al. Red blood cell microparticles show altered inflammatory chemokine binding and release ligand upon interaction with platelets. Transfusion. 2011;51:610–621. [PubMed: 20738825]
22. Gao Y, Lv L, Liu S, et al. Elevated levels of thrombin-generating microparticles in stored red blood cells. Vox Sang. 2013;105:11–17. [PubMed: 23347295]
23. Koshiar RL, Somajo S, Norström E, et al. Erythrocyte-derived microparticles supporting activated protein C-mediated regulation of blood coagulation. PLoS One. 2014;9:e104200. [PubMed: 25136857]
24. Belizaire RM, Prakash PS, Richter JR, et al. Microparticles from stored red blood cells activate neutrophils and cause lung injury after hemorrhage and resuscitation. J Am Coll Surg. 2012;214:648–655. [PubMed: 22342784]
25. Maślanka K, Uhrynowska M, Łopacz P, et al. Analysis of leucocyte antibodies, cytokines, lysophospholipids and cell microparticles in blood components implicated in post-transfusion reactions with dyspnoea. Vox Sang. 2015; 108:27–36. [PubMed: 25134637]
26. Gulbins E. Regulation of death receptor signaling and apoptosis by ceramide. Pharmacol Res. 2003;47:393–399. [PubMed: 12676513]
27. Dinkla S, Wessels K, Verdurmen WP, et al. Functional consequences of sphingomyelinase-induced changes in erythrocyte membrane structure. Cell Death Dis. 2012;3:e410. [PubMed: 23076218]
28. Awojoodu AO, Keegan PM, Lane AR, et al. Acid sphingomyelinase is activated in sickle cell erythrocytes and contributes to inflammatory microparticle generation in SCD. Blood. 2014;124:1941–1950. [PubMed: 25075126]
29. Peng H, Li C, Kadow S, et al. Acid sphingomyelinase inhibition protects mice from lung edema and lethal Staphylococcus aureus sepsis. J Mol Med (Berl). 2015;93:675–689. [PubMed: 25616357]
30. Wehrli G. Blood banking and transfusion medicine for the nephrologist. Semin Dial. 2012;25:114–118. [PubMed: 22276991]
31. György B, Szabó TG, Pásztói M, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci. 2011;68:2667–2688. [PubMed: 21560073]
32. Carpinteiro A, Becker KA, Japtok L, et al. Regulation of hematogenous tumor metastasis by acid sphingomyelinase. EMBO Mol Med. 2015;7: 714–734. [PubMed: 25851537]

33. Lawrie AS, Albanyan A, Cardigan RA, et al. Microparticle sizing by dynamic light scattering in fresh-frozen plasma. Vox Sang. 2009;96:206–212. [PubMed: 19175566]
34. Aatonen MT, Ohman T, Nyman TA, et al. Isolation and characterization of platelet-derived extracellular vesicles. J Extracell Vesicles. 2014;3.
35. Yuana Y, Bertina RM, Osanto S. Pre-analytical and analytical issues in the analysis of blood microparticles. Thromb Haemost. 2011;105:396–408. [PubMed: 21174005]
36. Chen Y, Li C, Weng D, et al. Neutralization of interleukin-17A delays progression of silica-induced lung inflammation and fibrosis in C57BL/6 mice. Toxicol Appl Pharmacol. 2014;275:62–72. [PubMed: 24291675]
37. Hardy RD, Coalson JJ, Peters J, et al. Analysis of pulmonary inflammation and function in the mouse and baboon after exposure to Mycoplasma pneumoniae CARDS toxin. PLoS One. 2009;4:e7562. [PubMed: 19859545]
38. Hestdal K, Ruscetti FW, Ihle JN, et al. Characterization and regulation ofRB6–8C5 antigen expression on murine bone marrow cells. J Immunol. 1991;147: 22–28. [PubMed: 1711076]
39. Makley AT, Goodman MD, Friend LA, et al. Murine blood banking: characterization and comparisons to human blood. Shock. 2010;34:40–45.
40. Kornhuber J, Tripal P, Gulbins E, et al. Functional inhibitors of acid sphingomyelinase (FIASMAs). Handb Exp Pharmacol. 2013;169–186. [PubMed: 23579455]
41. Lutz HU, Liu SC, Palek J. Release of spectrin-free vesicles from human erythrocytes during ATP depletion. I. Characterization of spectrin-free vesicles. J Cell Biol. 1977;73:548–560. [PubMed: 873988]
42. Bosman GJ, Lasonder E, Groenen-Döpp YA, et al. The proteome of erythrocyte-derived microparticles from plasma: new clues for erythrocyte aging and vesiculation. J Proteomics. 2012;76:203–210. [PubMed: 22669077]
43. Schissel SL, Jiang X, Tweedie-Hardman J, et al. Secretory sphingomyelinase, a product of the acid sphingomyelinase gene, can hydrolyze atherogenic lipoproteins at neutral pH. Implications for atherosclerotic lesion development. J Biol Chem. 1998;273:2738–2746. [PubMed: 9446580]
44. Kornhuber J, Rhein C, Müller CP, et al. Secretory sphingomyelinase in health and disease. Biol Chem. 2015;396:707–736. [PubMed: 25803076]
45. Esen M, Schreiner B, Jendrossek V, et al. Mechanisms of Staphylococcus aureus induced apoptosis of human endothelial cells. Apoptosis. 2001;6:431–439. [PubMed: 11595832]
46. Joint Theater Trauma System Clinical Practice Guideline: Fresh Whole Blood (FWB) Transfusion. U.S. Army Institute of Surgical Research, 2014 http://www.usaisr.amedd.army.mil/cpgs/Fresh_Whole_Blood_Transfusion_24_Oct_12.pdf. Accessed September 1, 2015.
47. Lheureux P, Vranckx M, Leduc D, et al. Flumazenil in mixed benzodiazepine/tricyclic antidepressant overdose: a placebo-controlled study in the dog. Am J Emerg Med. 1992;10:184–188. [PubMed: 1586424]
48. Hughes IE, Radman S. Relative toxicity of amitriptyline, imipramine, maprotiline and mianserin after intravenous infusion in conscious rabbits. Br J Clin Pharmacol. 1978;5(suppl 1):19S–20S. [PubMed: 623701]
49. Cleemput I, Leys M, Ramaekers D, et al. Balancing evidence and public opinion in health technology assessments: the case of leukoreduction. Int J Technol Assess Health Care. 2006;22:403–407. [PubMed: 16984672]
50. Kent MW, Kelher MR, West FB, et al. The pro-inflammatory potential of microparticles in red blood cell units. Transfus Med. 2014;24:176–181. [PubMed: 24786047]

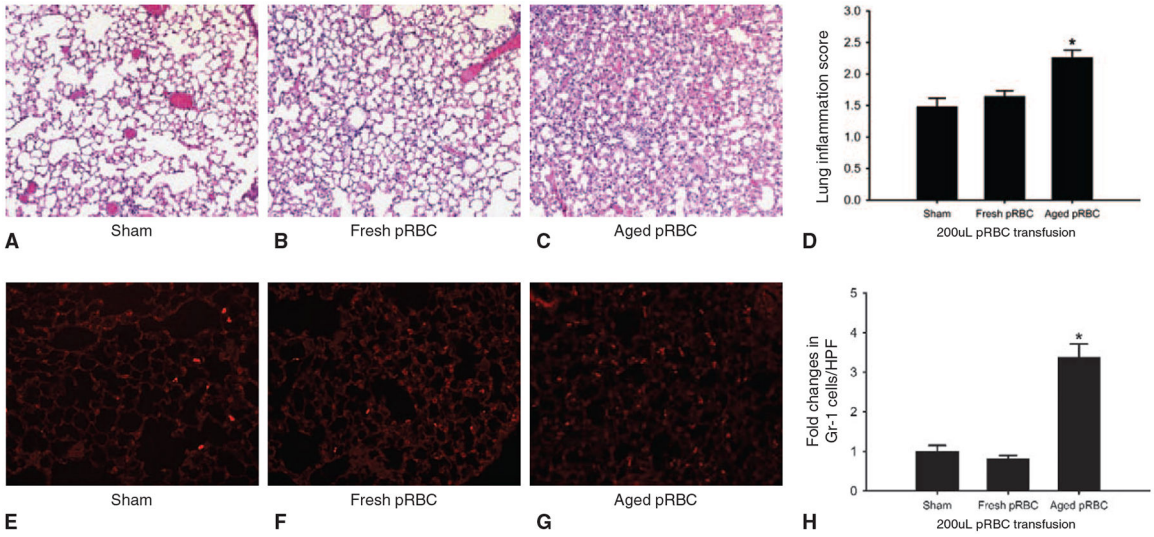

FIGURE 1.

Aged units of erythrocytes (pRBCs) cause lung inflammation. A–C, Representative H&E lung histology images from mice transfused with either no pRBCs (sham), fresh pRBCs, or pRBCs stored for 14 days (aged). Original magnification ×100. D, Composite lung inflammation score results from mice treated as described in images A–C (mean±SEM, n = 5 per group, $*P < 0.05$ vs sham and fresh pRBC groups, ANOVA). E–G, Immunofluorescence for Gr-1 in lungs of mice treated as in panels A–C. Original magnification ×400. H, Quantification of Gr-1 cells per high power field (mean±SEM, n = 5 per group, $*P < 0.05$ vs sham and fresh pRBCs, ANOVA).

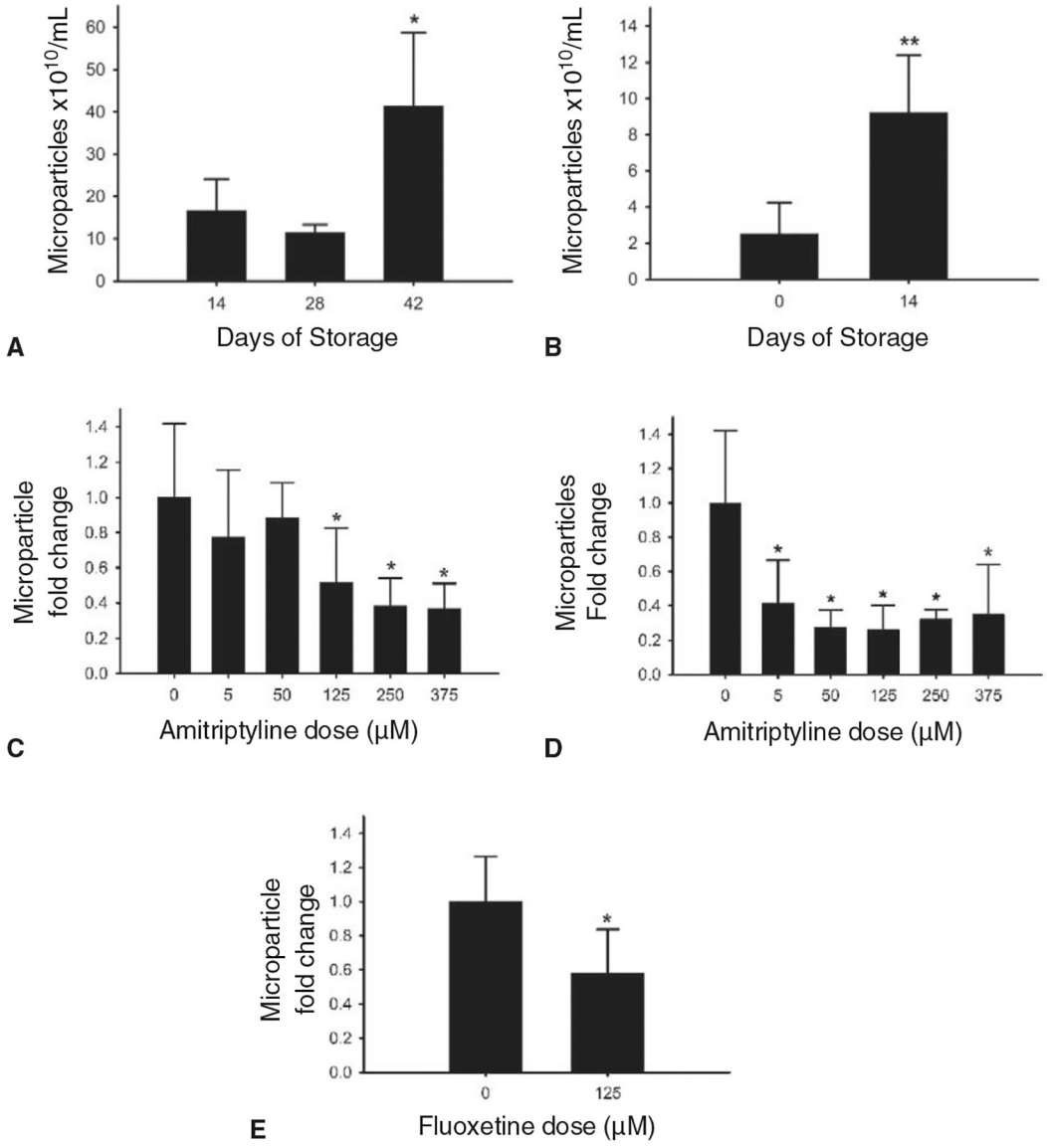

FIGURE 2.

Stored pRBCs generate microparticles, which is reduced by functional inhibition of acid sphingomyelinase. A, Microparticle concentrations in human pRBCs stored under standard storage conditions as determined by Nanoparticle Tracking Analysis (mean±SD, n = 7 per group, **P* < 0.05 vs days 14 and 28 groups, ANOVA). B, Microparticle concentrations in murine pRBCs stored for 14 days under standard storage conditions as determined by Nanoparticle Tracking Analysis (mean±SD, n = 4–6 per group, ***P* < 0.01 vs storage day 0, t test). Stored human (C) and mouse (D) pRBCs were treated with increasing doses of amitriptyline in normal saline (0 μM represents vehicle only). E, Mouse pRBCs were also treated with fluoxetine and microparticle counts measured using Nanoparticle Tracking Analysis. Results represent fold changes in microparticle counts with regards to the vehicle group (mean±SD, n = 5–7 per group, **P* < 0.05 compared with 0 μM group, ANOVA for A–B, *t* test for C).

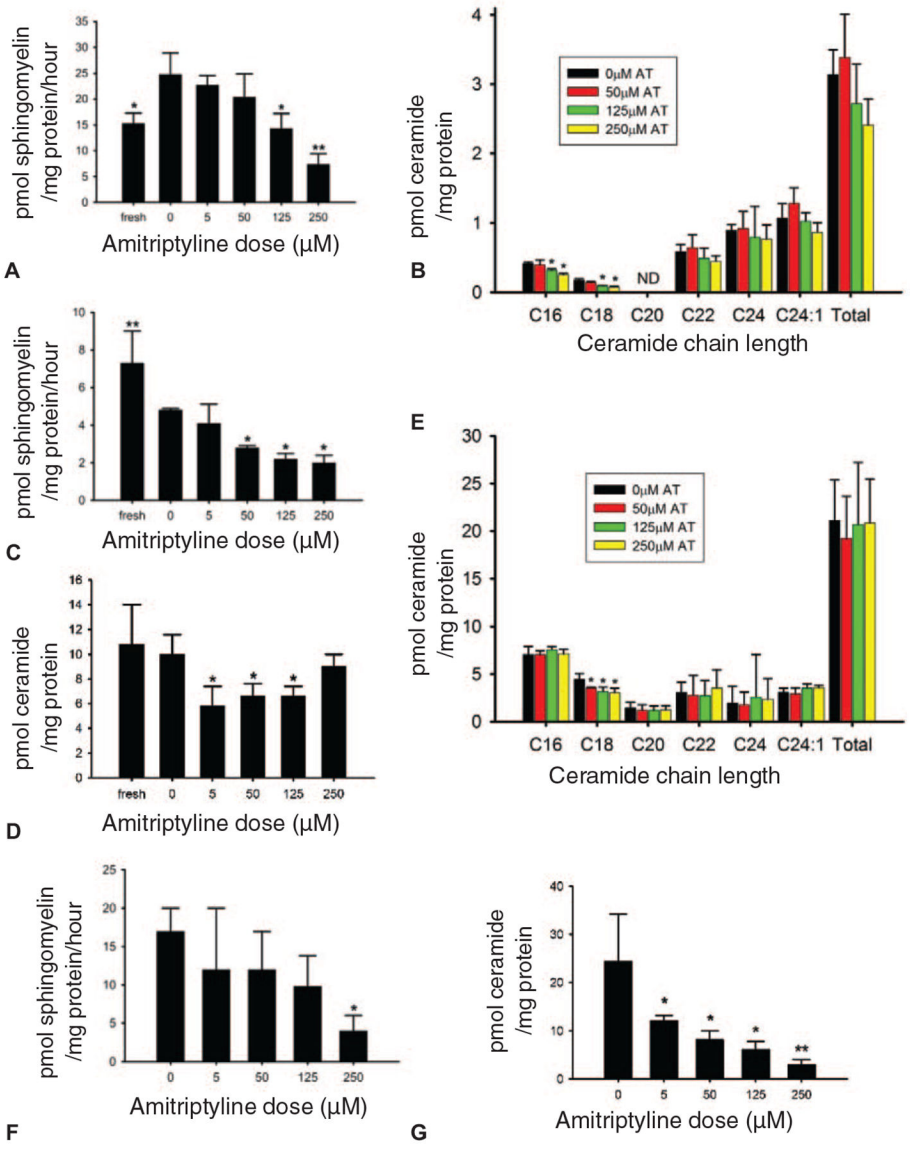

FIGURE 3.

Storage of pRBCs with amitriptyline reduces acid sphingomyelinase activity and ceramide formation in stored human and mouse pRBCs in a dose-dependent fashion. A, Microparticles in aged murine pRBCs (0 μM, vehicle-treated) show increased acid sphingomyelinase activity compared with those from fresh pRBCs, and this activity is reduced with amitriptyline treatment. B, Mass spectrometry analysis of these microparticles revealed a reduction in ceramide concentration as well, most notably C16 and C18 (ND indicates not detectable). C, Acid sphingomyelinase activity on aged erythrocytes is reduced compared with fresh erythrocytes, and this activity is further reduced by amitriptyline. D, E, Ceramide concentrations on aged erythrocytes are decreased with amitriptyline treatment, specifically C18. Measurements were taken at day 7 of storage (mean±SD, n = 5 per group, $*P < 0.05$ vs 0 μM, $**P < 0.05$ vs all groups, ANOVA). F, G, Microparticles isolated from aged human pRBCs demonstrate a similar reduction in both acid sphingomyelinase activity

(F) and overall ceramide concentration (G) with amitriptyline treatment (measurements taken at day 28 of storage, mean±SD, n = 5 group, *$P < 0.05$ vs 0 μM, **$P < 0.05$ vs all groups, ANOVA).

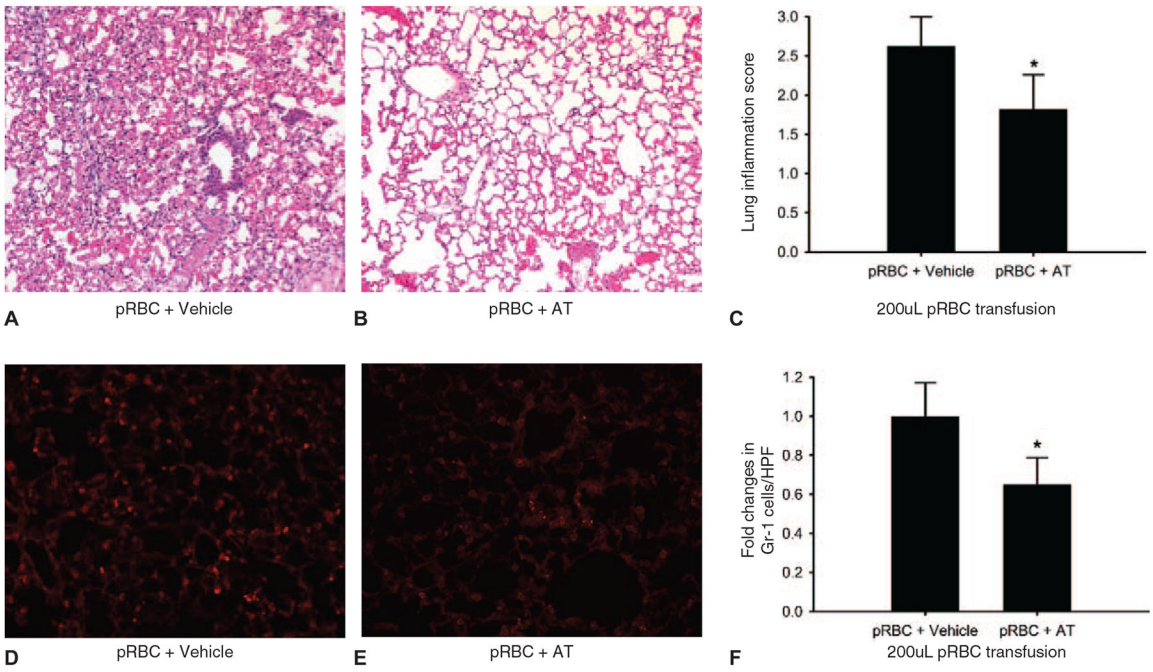

FIGURE 4.

Storage of pRBCs with amitriptyline reduces lung inflammation after transfusion. Mice which received 200 μL transfusions of pRBCs treated with amitriptyline demonstrate decreased lung inflammation on H&E (A, B), decreased composite lung inflammation scores (C), and decreased Gr-1 positive cell presence in the lungs (D–F) (mean±SEM, n = 5 per group, $*P < 0.05$, *t* test). H&E images were taken at original magnification ×100 and Gr-1 immunofluorescence images were taken at original magnification ×400.

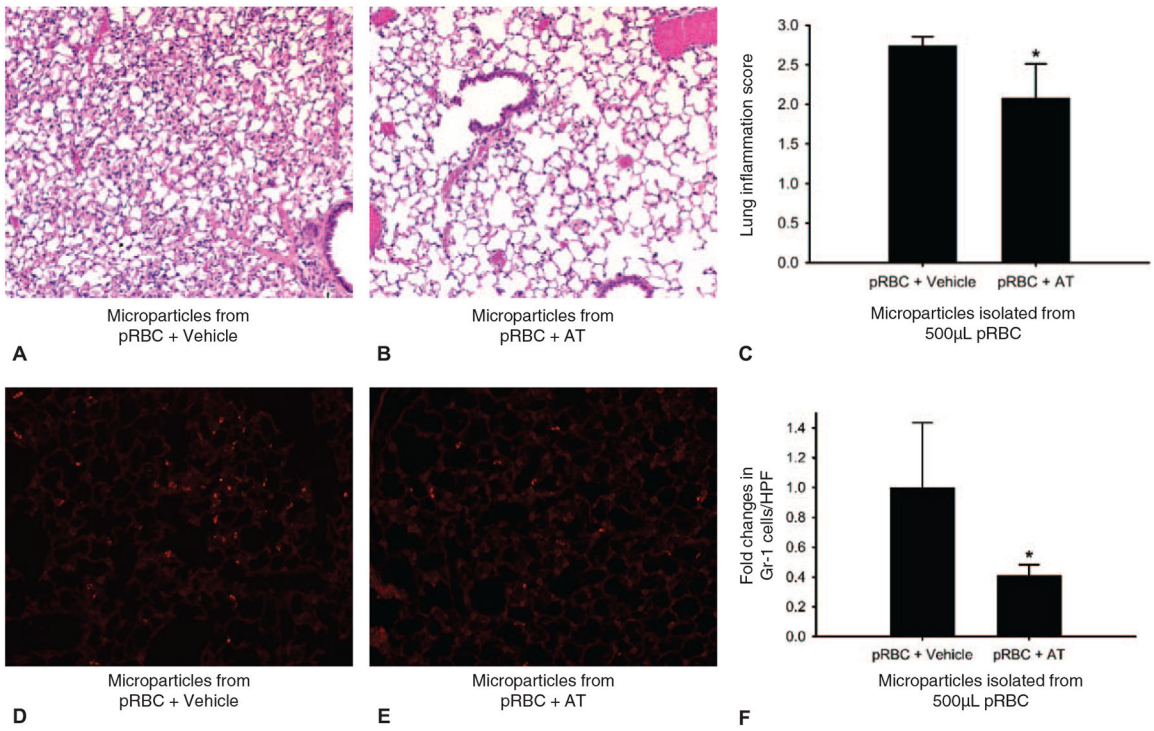

FIGURE 5.

Microparticles isolated from pRBCs stored with amitriptyline cause less lung inflammation than those from untreated pRBCs. Microparticles from 0.5 mL pRBC were isolated and resuspended in normal saline for IV injection. Mice who received microparticles from pRBCs stored with amitriptyline had significantly reduced lung inflammation (A–C) and GR-1 positive cells (D–F) compared with those receiving microparticles isolated from untreated pRBCs (mean±SEM, n = 5 per group, $*P < 0.05$, *t* test). H&E images were taken at original magnification ×100 and Gr-1 immunofluorescence images were taken at original magnification ×400.

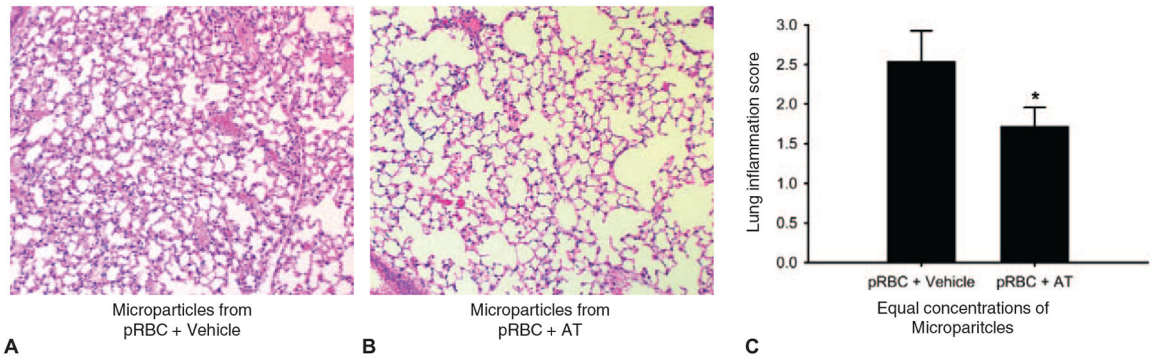

FIGURE 6.

Equal concentrations of microparticles from pRBCs stored with amitriptyline cause less lung inflammation than those from pRBCs stored with vehicle. Microparticles from 0.5 mL vehicle-treated pRBCs were isolated and resuspended in normal saline at equal concentration to microparticles prepared from 0.5 mL pRBCs stored with amitriptyline. Mice which received microparticles from vehicle-treated pRBCs (A) had significantly increased lung inflammation compared with those receiving microparticles isolated from pRBCs stored with amitriptyline (B, C) (mean±SEM, n = 5 per group, $*P < 0.05$, *t* test). H&E images were taken at original magnification ×100.

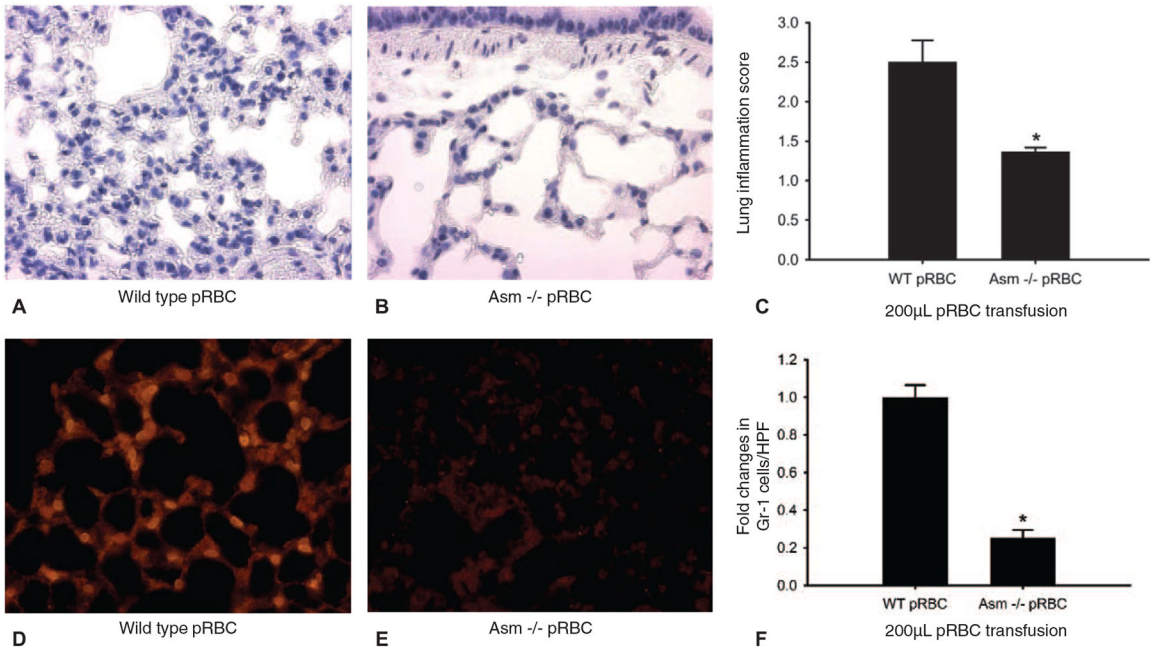

FIGURE 7.

pRBCs from acid sphingomyelinase knockout ($ASM^{-/-}$) mice cause less lung inflammation in transfusion recipients than those from wild-type (WT) mice. Mice which received 200 μL transfusions of pRBCs from ASM-deficient ($ASM^{-/-}$) mice demonstrate decreased lung inflammation on H&E. (A, B), decreased composite lung inflammation scores (C), and decreased Gr-1 positive cell presence in the lungs (D–F) (mean±SEM, n = 6 per group, $*P < 0.05$, *t* test). H&E were taken at original magnification ×100 and Gr-1 immunofluorescence images were taken at original magnification ×400.