Plasma Transfusion Products and Contamination with Cellular and Associated Pro-Inflammatory Debris

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BACKGROUND: Stored plasma products are widely regarded as being functionally acellular, obviating the need for leukoreduction. We tested the hypothesis that donor plasma is contaminated by leukocytes and platelets, which, after frozen storage, would release cellular debris in quantities sufficient to elicit significant pro-inflammatory responses.

STUDY DESIGN: Samples of never-frozen liquid plasma from 2 regional Level I trauma centers were analyzed for leukocyte and platelet contamination. To determine if the cellular contamination and associated debris found in liquid plasma were at levels sufficient to evoke an innate immune response, known quantities of leukocytes were subjected to a freeze-thaw cycle, added to whole blood, and the magnitude of the inflammatory response was determined by induction of interleukin-6.

RESULTS: Units of never-frozen plasma from 2 regional Level I trauma centers located in Alabama and Louisiana contained significant amounts of leukocyte contamination (Louisiana, $n = 22$; 17.3 ± 4.5 million vs Alabama, $n = 22$; 11.3 ± 2.2 million) and platelet contamination (Louisiana, $n = 21$; 0.86 ± 0.20 billion vs Alabama, $n = 22$; 1.0 ± 0.3 billion). Cellular debris from as few as 1 million leukocytes induced significant increases in interleukin-6 levels ($R^2 = 0.74$; $p < 0.0001$).

CONCLUSIONS: Stored plasma units from trauma center blood banks were highly contaminated with leukocytes and platelets, at levels more than 15-fold higher than sufficient to elicit ex vivo inflammatory responses. In light of paradigm shifts toward the use of more empiric plasma for treatment of hypovolemia, this study suggests that new manufacturing and quality-control processes are needed to eliminate previously unrecognized cellular contamination present in stored plasma products. (J Am Coll Surg 2019;229:252–258. © 2019 by the American College of Surgeons. Published by Elsevier Inc. All rights reserved.)

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A recent paradigm shift in transfusion strategies for hemorrhagic shock includes the transfusion of stored blood components in equal ratios in an effort to reconstitute whole blood. This change in the standard of care has resulted in large increases in the quantity of plasma products being transfused to critically ill patients.[1] Despite a substantial decrease in trauma patient mortality from hemorrhagic shock, there is a lack of understanding about the pathophysiologic consequences of modern massive transfusion protocols. Traditionally, transfusion medicine has focused on interactions among blood group antigens, antibodies, and the immune system; however, multiple lines of evidence point to additional active mediators found within human blood components, including

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Abbreviations and Acronyms
DAMP = damage-associated molecular pattern
FFP = fresh-frozen plasma
IL = interleukin
LPS = lipopolysaccharide
PBS = phosphate buffered saline
PF-24 = plasma frozen within 24 hours of collection

damage-associated molecular patterns (DAMPs).[2] Recent studies have demonstrated a direct correlation between these active mediators and the development of transfusion-related acute respiratory distress syndrome.[3]

The deleterious effects of leukocyte contamination in cellular blood components, such as RBCs and platelets, is well understood and tightly quality-controlled.[4] Several methods of leukoreduction have been developed with the goal of eliminating leukocyte contamination from both RBC and platelet units.[5-7] Conceptually, stored plasma products are widely regarded by physicians as being acellular, obviating the need for leukoreduction as a standard part of plasma processing. However, previous studies have revealed a large discrepancy in the quantity of inflammatory cellular debris contained in fresh-frozen plasma (FFP) compared with leukocyte-reduced packed RBCs, suggesting the possibility that some sources of FFP contain large quantities of leukocytes.[8] Any contaminating leukocytes present in direct-from-patient donated plasma are ruptured by the freezing process of FFP storage, impeding the ability to detect cellular contamination in FFP as an end product. In addition to masking the potential existence of cellular contamination, the freeze-thaw cycle required for plasma storage would also result in direct cellular rupture of any contaminating cells with release of pro-inflammatory intracellular organelles and debris.

Reflecting their bacterial ancestry, eukaryotic organelles and associated contents are highly pro-inflammatory when released to the extracellular milieu; complex systems for sequestering and eliminating these moieties have evolved into multiple mechanisms for programmed cell death.[9] Pro-inflammatory self-molecules that escape these control mechanisms and are released into systemic circulation are termed *damage-associated molecular patterns*.[10,11] Several recent reports have associated circulating DAMPs with systemic inflammatory responses and the propagation of multi-organ failure in a number of clinical scenarios.[3,11-13] In particular, the presence of FFP and platelet contamination with mitochondrial DAMPs was recently shown to be associated with the development of transfusion-related acute respiratory distress syndrome.[3] As the only commonly used blood component that is routinely stored frozen and thawed for use, contamination of plasma with cellular and organellar debris would likely lead to inflammatory responses when introduced into patients, although this has never been investigated directly. Against this background, we tested the hypothesis that liquid plasma is contaminated by leukocytes and platelets, which, on processing into FFP, release DAMPs in quantities sufficient to activate the innate immune system.

METHODS

Determining cellular contamination of never-frozen liquid plasma

Samples of male-donor never-frozen liquid plasma (n = 44) were obtained from the blood banks of two large Level I trauma centers, including the University of South Alabama (Mobile, AL) and the Norman E McSwain Jr Spirit of Charity Trauma Center (New Orleans, LA). The methods of plasma production used by the institutions reflect the industry standard. All samples were obtained from tubular segments attached to each stored blood component, which are routinely set aside during the procurement process for testing purposes. All units were created by single-step centrifugation and were not leukocyte-reduced, representing standard units available for routine transfusion in each hospital's blood bank. All samples were transported by the research team to ensure proper temperature conditions and to avoid introducing freeze-thaw events before testing.

All samples were analyzed in the University of South Alabama clinical laboratory with flow cytometry (Sysmex XN). Leukocyte count was obtained by analyzing the samples via body fluid mode, and platelet number was obtained via platelet fluorescence functionality. The manufacturer stated linearity and the institution quality-control data were reviewed to ensure result reliability. Acceptable parameter ranges for leukocytes and platelets fall between 0 to 440 cells/μL and 0 to 5,000 cells/μL, respectively.[14] All experimental results fell within acceptable ranges for instrument linearity. Manual cell counts were performed as an additional method of verification using cell chamber methods with light microscopy. The raw data in number of cells per microliter of sample were then extrapolated to represent total cell number in millions of cells per unit of blood product for leukocytes and in billions of cells per

unit of blood product for platelets. This was determined by fixing the volume at 300 mL per unit, which reflects the mean volume of plasma product per unit produced by the regional blood center LifeSouth, which supplies blood products to the University of South Alabama.

Determining a dose-response curve for cellular debris-induced inflammation

To determine whether the cellular contamination and associated debris found in liquid plasma were at levels sufficient to evoke an innate immune response, we developed an immune activation assay. The goal was to model conditions seen in FFP, where cellular debris originates from the rupture of leukocytes after a freeze-thaw cycle. Known quantities of leukocytes were subjected to a freeze-thaw cycle, added to whole blood, and the magnitude of the inflammatory response was determined. Here we assayed for interleukin (IL)-6 production, as it is a well-studied mediator of acute-phase inflammatory responses and has a demonstrated negative relationship with outcomes in critically ill patients.

Regulatory control of the innate immune system engages multiple signaling pathways before initiating a systemic inflammatory response to prevent an inappropriate activation of the immune system. In developing the assay, we rationalized that critically ill patients receiving transfusions would already be in an immunologically primed state. Therefore, before adding freeze-thaw debris, we primed the whole blood with 250 ng/mL *Escherichia coli* lipopolysaccharide (LPS) (O127:B8; Millipore Sigma) to engage the Toll-like receptor 4 pattern recognition signaling pathway. Activation of Toll-like receptor 4 primes the nuclear factor-κB signaling pathway in innate immune cells to upregulate and secrete cytokines, such as tumor necrosis factor-α, IL-1β, IL-8, and IL-6, which induce an inflammatory response. The expectation here was that addition of cellular debris present in the freeze-thaw debris would augment cytokine production.

To generate cellular debris, leukocytes from the buffy coat of type A whole blood (ZenBio) were enumerated using fluorescent flow cytometry (Sysmex XN). The leukocytes were then divided into aliquots of 10^5, 10^6, and 10^7 cells. Each aliquot was diluted with phosphate buffered saline (PBS, pH 7.2) and frozen at -80°C for 24 hours. Next, the assay was prepared by diluting whole blood (ZenBio) in a 1:4 ratio (1 mL whole blood to 3 mL medium) with RPMI-1640 medium (Millipore Sigma). After thawing, each cell aliquot was added to the whole blood assay and the mixture was then incubated for 24 hours at 37°C with 5% CO_2 on 6-well culture plates (ThermoFisher). Whole blood (no LPS) with added PBS served as a negative control. As a positive control, whole blood primed with LPS with added PBS was used to generate IL-6 production, indicative of the baseline inflammatory potential of the system. After incubation, the contents were recovered from the culture plates and cells removed by centrifugation at 2,000*g* for 10 minutes at ambient temperature. Supernatant fractions were recovered and stored frozen at -20°C until cytokine measurements were performed. The IL-6 concentration was measured in the culture supernatant by ELISA, in accordance with the manufacturer's protocol (Qiagen).

Statistical analysis

Data were analyzed using Prism 8 software (GraphPad). Quantitative data are presented as mean ± SEM. Differences between experimental groups were determined with a 1-way ANOVA with Newman-Keuls post-hoc test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Never-frozen liquid plasma is highly contaminated by leukocytes and platelets

Available units of never-frozen plasma from Alabama (n = 22) and Louisiana (n = 22) were analyzed to quantify the number of leukocytes and platelets in each unit. The samples from the University of South Alabama contained a mean of 11.3 ± 2.2 million leukocytes (Fig. 1) and 1.0 ± 0.3 billion platelets per 300 mL (Fig. 2). The samples from Louisiana also displayed large quantities of cellular contamination, including a mean of 17.3 ± 4.5 million leukocytes (Fig. 1) and 0.9 ± 0.2 billion platelets per each 300 mL (Fig. 2). The quantity of leukocytes ($p = 0.7$) and platelets ($p = 0.8$) did not differ significantly between institutions. Pearson correlation was performed between platelets and leukocytes, but interestingly did not reveal a significant correlation ($r = 0.02$, $p = 0.9$). Of note, 1 sample showing a platelet value of 34,000 cells/μL (102 billion platelets per 300 mL) was deemed to be a significant outlier and was therefore excluded from the study. These data suggest that plasma-manufacturing processes at 2 different blood banks are insufficient to prevent significant contamination with leukocytes and platelets.

Cellular debris evokes an inflammatory response in whole blood

After thawing, any cells and platelets in FFP would rupture and the debris would be recognized by the innate immune system, simulating a deleterious inflammatory response in a patient post transfusion. The addition of

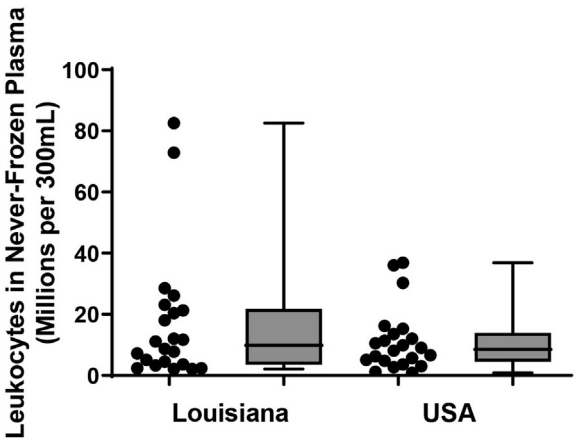

Figure 1. Never-frozen plasma contains large quantities of leukocytes. Units of never-frozen plasma from the University of South Alabama (right) and the Norman E McSwain Jr Spirit of Charity Trauma Center (left) contained significant amounts of leukocyte contamination (Louisiana, n = 22; 17.1 ± 4.5 million vs Alabama, n = 22; 11.3 ± 2.2 million). The amount of leukocyte contamination between centers was similar (p = 0.70). Individual samples from each institution are expressed as a single data point (scatter plot, left) and as median, quartiles, and range (box and whiskers, right).

cellular debris to a critically ill patient in an immunologically primed state might evoke an exacerbated inflammatory response, leading to poor outcomes. To simulate the effects of freeze-thaw cycles on FFP, leukocytes were isolated from whole blood; enumerated; diluted to final cell numbers of 10^5, 10^6, and 10^7; and frozen overnight (see details in Methods section). Cellular debris was generated by thawing the cell aliquots, which were subsequently added to whole blood with and without LPS priming. The whole blood was primed with LPS to induce a baseline level of Toll-like receptor 4-mediated IL-6 production and secretion to mimic the immunologically primed state of a critically ill patient. Secreted IL-6 levels were measured 24 hours later to determine the magnitude of inflammatory response.

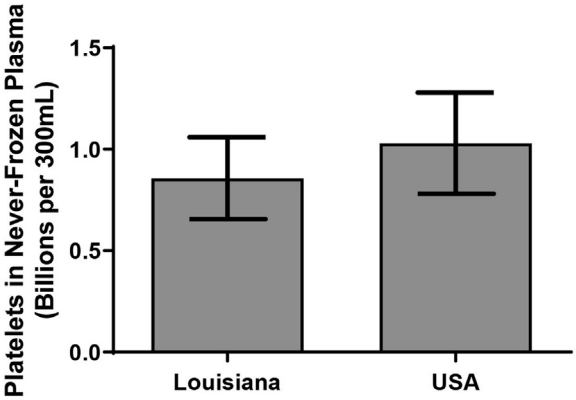

Figure 2. Never-frozen plasma contains large quantities of platelets. Units of never-frozen plasma from the University of South Alabama (left) and the Norman E McSwain Jr Spirit of Charity Trauma Center (right) contained significant amounts of platelet contamination (Louisiana, n = 21; 0.9 ± 0.2 billion vs Alabama, n = 22; 1.0 ± 0.3 billion). The amount of platelet contamination between centers was similar (p = 0.8). Data in this figure are represented by means ± SEM in billions per 300-mL unit of plasma.

As expected, negative control samples consisting of whole blood and PBS (no cellular debris) did not demonstrate measurable IL-6 secretion. In addition, whole blood (no LPS) with added cellular debris did not induce IL-6 secretion. An experimental baseline of IL-6 secretion was established by adding LPS to whole blood in the absence of cellular debris. The IL-6 levels remained unchanged by the addition of cellular debris from 10^5 leukocytes subjected to a freeze-thaw cycle compared with LPS treatment alone (baseline). However, addition of cellular debris from 10^6 and 10^7 leukocytes to LPS-primed whole blood revealed significant increases in IL-6 secretion (Figs. 3A, 3B) ($R^2 = 0.70$, $p < 0.0001$). The IL-6 concentrations were normalized to the average positive control of each experiment (whole blood primed with LPS in the absence of cellular contamination). The normalized means ± SEMs for IL-6 production are as follows: negative control (no LPS, no leukocytes) 0.00 ± 0.00, positive control (LPS, no leukocytes) 0.99 ± 0.24, 10^5 leukocytes 0.90 ± 0.04, 10^6 leukocytes 1.30 ± 0.04, 10^7 leukocytes 1.49 ± 0.08 (Fig. 4). Together these data suggest that the quantities of cellular contamination and associated debris that are known to be present in a single unit of liquid plasma from 2 different blood banks are capable of activating the innate immune system, suggesting clinical significance.

DISCUSSION

Blood banking practices, including production of blood components from whole blood in the US, are regulated by the Food and Drug Administration and accredited by the AABB. These entities have recognized the implications of leukocyte contamination in RBCs, with universal leukocyte reduction now being the standard for most hospitals. Donor leukocytes in RBC and platelet units are linked to a variety of acute and chronic complications and thereby removed via filtration leukoreduction to concentrations $<5 \times 10^6$ total cells per unit.[15] As visual buffy coat exclusion during plasma extraction is believed to adequately avoid cellular contamination, filter leukoreduction is not routinely performed on plasma before processing into FFP. Therefore, stored plasma products are not to be tested for residual leukocyte contamination,

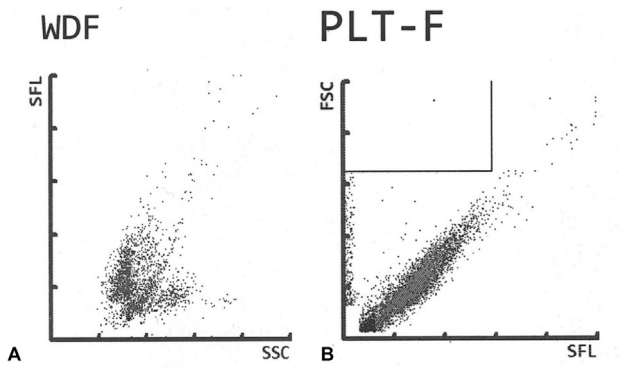

Figure 3. Flow cytometry (Sysmex XN) results demonstrating cellular contamination from leukocytes and platelets in liquid plasma. (A) Scattergram depicting fluorescence flow cytometry for leukocytes in liquid plasma by the WBC differential channel (WDF). The scattergram is a composite measuring side-scattered light signal (SSC) along the x-axis and side-fluorescence light signal (SFL) along the y-axis. (B) Scattergram depicting fluorescence flow cytometry for platelets in liquid plasma by the platelet fluorescence channel (PLT-F). The scattergram is a composite measuring SFL along the x-axis and forward-scattered light signal (FSC) along the y-axis.

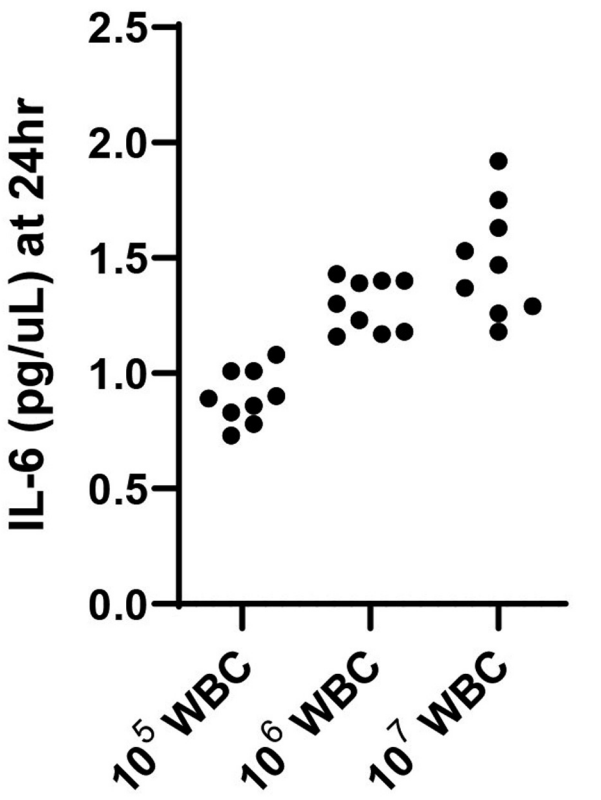

Figure 4. Debris from as few as 1 million cells was sufficient to elicit a pro-inflammatory response. Three quantities of leukocytes were frozen and thawed before adding each to whole blood. These experiments show the debris from as few as 1 million leukocytes is sufficient to create an inflammatory response within whole blood at 24 hours ($R^2 = 0.70$, $p < 0.0001$).

which is required in leukocyte-reduced packed RBCs and platelet-rich plasma.

Current manufacturing processes that create plasma products from centrifugation of whole blood separate the relative components by specific gravity. After centrifugation, plasma is expressed manually by technicians based on visual assessment of the transition between plasma and the buffy coat, the first cell layer that contains leukocytes and platelets. As multiple samples are simultaneously processed by a single operator and the buffy coat has varying levels of distinctness in different samples, this is a potential source of cellular contamination. Unlike cellular blood components, such as RBCs and platelets, no additional testing of the product for leukocytes or platelets occurs. Additionally, the lack of correlation between leukocytes and platelet contamination suggests that the centrifugation process can be an additional source of contamination and might account for some of the variability in the extent of cellular contamination described in this study.

Frozen plasma products, such as FFP (plasma frozen within 8 hours of collection) and plasma frozen within 24 hours of collection (PF-24) are the most frequent forms of transfused plasma in the US because of their long shelf-life. Previous studies examining cellular contamination in thawed plasma (ie FFP and PF-24) are limited in their ability to accurately quantify the number of leukocytes, as the freeze-thaw process ruptures most cells.[16-18] Evolving practice patterns at many high-volume trauma centers now include the use of never-frozen liquid plasma because of its immediate availability for transfusion. Never-frozen liquid plasma is manufactured in an identical manner to FFP and PF-24, minus the step of freezing the solution for storage. As the product is never frozen, it is now possible to accurately measure intact cells in plasma products originating from whole blood centrifugation. Herein, we found considerable cellular contamination in 44 units of never-frozen liquid plasma from blood banks located in Alabama and Louisiana. Cellular contamination varied between 0.9 and 82.5 million cells per unit for leukocytes and between 0 and 4.9 billion cells per unit for platelets.

The lysis of eukaryotic cells and subsequent release of self-molecules is far from innocuous. Release of DAMPs into the extracellular milieu is a potent activator of the innate immune system, as first described in 1994,[19] but was not clinically implicated until more recently.[12,20] Current evidence that DAMPs contribute to human disease is

compelling.[3,21] Related to the current study, previous reports revealed large variations in mitochondrial DNA DAMPs within FFP compared with leukocyte-reduced RBC units, but a clinically relevant threshold level of cellular contamination has not been established.[8] Through a series of experiments, cellular debris from as few as 1 million cells is sufficient to create an inflammatory response ex vivo, signifying leukoreduction below this level might be necessary. Indeed, critically ill patients often receive multiple units of plasma, highlighting the importance of strategies to eliminate cellular contamination from stored plasma.

The implications of this study are limited by some important considerations. This study only considered plasma created by whole blood centrifugation, as it represents the majority of units available for transfusion. The amount of cellular contamination in plasma created by apheresis is unexplored and can differ, given its unique manufacturing process. Although there is wide agreement that cellular debris are pro-inflammatory, the amount of inflammation attributed to each individual cellular DAMP remains uncharacterized. This becomes highly relevant, considering the current study reveals large quantities of both platelets and leukocytes within stored plasma. Finally, the diagnosis leading to critical illness can induce physiologic priming of the patient's immune system in varying degrees, thereby causing the inflammatory effects of transfused cellular debris to also vary in severity.

CONCLUSIONS

Stored plasma units from trauma center blood banks were highly contaminated with leukocytes and platelets, at levels more than 15-fold higher than sufficient to elicit ex vivo inflammatory responses. This level of cellular contamination can be associated with significant deleterious effects in plasma-transfused patients. In light of paradigm shifts toward the use of more empiric plasma for treatment of hemorrhagic shock, this study suggests that new manufacturing and quality-control processes are needed to eliminate previously unrecognized cellular contamination present in stored plasma products.

Author Contributions

Principal investigator: Simmons
Study conception and design: Tan, Audia, Simmons
Acquisition of data: Tan, Rieske, Simmons
Analysis and interpretation of data: Tan, Rieske, Simmons
Drafting of manuscript: Tan, Rieske, Simmons
Critical revision: Tan, Rieske, Audia, Pastukh, Capley, Gillespie, Smith, Tatum, Duchesne, Kutcher, Kerby, Simmons

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