Immunomodulatory effects of plasma products on monocyte function in vitro

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BACKGROUND: Restoration of a balanced innate immune response is paramount to recovery from critical injury. Plasma transfusion may modulate innate immune responses; however, little is known about the immunomodulatory potential of various plasma products. We conducted in vitro experiments to determine the effects of fresh frozen plasma, thawed plasma, solvent/detergent plasma, and an investigational spray-dried solvent/detergent plasma product on monocyte function.

METHODS: Monocytes were isolated from healthy adult volunteers and cocultured with aliquots of autologous plasma (control), fresh frozen plasma, thawed plasma, solvent/detergent treated plasma, or spray-dried solvent/detergent plasma. Monocyte function was assessed by cytokine production with and without lipopolysaccharide (LPS) stimulation, and flow cytometric assessment of HLA-DR cell surface expression.

RESULTS: Monocyte cytokine production was not significantly altered after exposure to fresh frozen plasma or thawed plasma. In the absence of LPS, spray-dried solvent/detergent plasma exposure resulted in markedly increased IL-8 production compared to other plasma groups and controls ($p = 0.01$, analysis of variance [ANOVA]). Likewise, spray-dried SD plasma exposure resulted in higher LPS-induced IL-8, TNFα, and IL-1β production compared with autologous plasma controls ($p < 0.0001$; $p < 0.0001$, $p = 0.002$, respectively; ANOVA). LPS-induced IL-8 and TNFα production was lowest after exposure to solvent/detergent plasma ($p < 0.0001$, ANOVA).

CONCLUSION: Exposure to spray-dried solvent/detergent plasma resulted in marked augmentation of monocyte inflammatory cytokine production. Solvent/detergent plasma exposure resulted in the lowest cytokine production, suggesting lower immunomodulatory potential. Further work is needed to determine how these in vitro findings may translate to the bedside. (*J Trauma Acute Care Surg*. 2018;84: S47–S53.

KEY WORDS: Trauma; immune; plasma; transfusion; monocyte.

Plasma transfusion is common in critically ill and injured patients, with nearly 4 million units of plasma transfused annually in the United States.[1] Particularly in the setting of critical trauma and massive transfusion, plasma products may be lifesaving.[2–4] Yet, plasma transfusion is also independently associated with adverse outcomes in critical illness, including increased odds of acute lung injury/acute respiratory distress syndrome, nosocomial infection, and multiple organ failure.[5–8] Mechanisms underlying the relationships between plasma transfusion and adverse outcomes in the critically ill and injured are unclear, though they may include alterations in recipient immune function.

Innate immune dysfunction, characterized by concurrent systemic inflammation and severe immune suppression, is recognized as an important risk factor for adverse outcomes in critically ill and injured patients.[9–16] Causes of innate immune dysfunction in critical illness and injury remain unclear, though previous studies implicate blood product transfusions as contributing factors.[17–20] Because most previous studies of immune modulation related to transfusion focus on red blood cell products, much less is known about immunomodulatory effects of plasma products.

Several different plasma products are currently in use or under production in the United States. Products currently in use include single-donor fresh frozen plasma (FFP), which is separated from whole blood and frozen within 8 hours of collection; PF-24, which is plasma separated from whole blood and frozen within 24 hours of collection; and thawed plasma, which is FFP or PF-24 which has been thawed and stored at 4°C to 6°C for up to 5 days before transfusion. These plasma preparations are often used interchangeably and represent 37%, 27%, and 30% of total units transfused in the United States, respectively.[1] In 2013, a solvent/detergent-treated pooled plasma product, Octaplas (Octapharma Hoboken, NJ) was licensed by the FDA and became available in the US. Additionally, a spray-dried solvent/detergent plasma product, Resussix (Entegrion, Research Triangle Park, NC) is currently under development by the Office of Naval Research and the Department of Defense. A previous study demonstrated important differences in potentially immunomodulatory mediators, including microparticles and residual cells, across these different plasma preparations.[21] Thus, it is likely that immunomodulatory effects of each of these products

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may differ. However, effects of individual plasma products on monocyte function have not been previously studied and remain unknown.

We therefore used an established in vitro transfusion model to test the hypothesis that plasma products will differentially modulate monocyte function in vitro.

METHODS

Blood Collection and Monocyte Isolation

The study protocol was reviewed by the institutional review board at Nationwide Children's Hospital and was determined by the IRB to not be human subject research because no clinical data were collected for the purposes of analysis and cells were not used to create cell lines. For monocyte isolation, up to 110 mL of blood was drawn from healthy adult volunteers in ethylenediaminetetraacetate tubes. Donors were excluded if they had a history of cancer or post-transplant status, or were receiving immunosuppressive or anti-inflammatory medications (including the use of aspirin or nonsteroidal anti-inflammatory medications within the past 48 hours). Monocytes were isolated within 30 minutes following blood draws and were used immediately in coculture models. Monocytes were isolated as previously described.[18,22] Briefly, whole blood was diluted 1:1 with phosphate buffered saline, and peripheral blood mononuclear cells were then collected by density gradient centrifugation using lymphocyte separation medium (Mediatech, Manassas, VA). Monocytes were further purified by positive selection using CD14 magnetic beads (Miltenyi Biotec, Auburn, CA) and were resuspended in Roswell Park Memorial Institute (RPMI) Medium. Percent purity using this method is at least 98% as previously reported.[23]

Whole blood for autologous plasma was collected concurrently into heparin tubes from the same monocyte donor. Heparin tubes were used for autologous plasma collection as opposed to citrate tubes to not mask potential immunomodulatory effects of citrate content in plasma products. Autologous plasma was obtained by centrifugation at 1,000 times gravity for 5 minutes at 4°C.

Plasma Product Preparation and Coculture Models

Fresh frozen plasma units were obtained from the blood bank at Nationwide Children's Hospital. Solvent/detergent (SD) plasma units were obtained from Octapharma. For each experiment, FFP and SD plasma units were thawed in the blood bank at Nationwide Children's hospital using standard protocol. Immediately after thawing, aliquots were obtained using a sterile connection device and blood administration syringe sets (CODAN, Santa Ana, CA). Remaining thawed FFP units were stored at 4°C to 6°C for 4 days to 5 days before aliquoting for thawed plasma. Spray-dried SD plasma and pre-spray dried SD plasma units were obtained from Entegrion. For each experiment, spray-dried SD plasma was reconstituted in manufacturer provided reconstitution solution according to manufacturer protocol. All plasma products were from blood type A, Rh + donors.

In Vitro Transfusion Model and Cytokine Measurements

Coculture models were adapted from our previously published in vitro transfusion model.[18,22] Experimental timeline for in vitro studies is shown in Supplemental Figure 1 (see Figure, Supplemental Digital Content 1, http://links.lww.com/TA/B86). For each replicate of experiments, one million monocytes per well were plated onto pyrogen-free 12-well plates and cocultured in RPMI with 40% by volume autologous plasma (control), FFP, thawed plasma, SD plasma, or spray-dried SD plasma. Forty % by volume was chosen based on dose response experiments and to simulate volumes of plasma given under massive transfusion protocols.[4] Cells were incubated for 18 hours at 37°C. After this incubation period, cells were stimulated with LPS 1 ng/mL (phenol-extracted from *Salmonella abortus* equii; Sigma, St Louis, MO) to determine LPS-induced cytokine production capacity. Plates were then incubated for an additional 4 hours at 37°C. Cell culture supernatant from each well was collected, centrifuged, and stored at −80°C for subsequent cytokine analysis. Pro-inflammatory cytokines TNFα, IL-1β, and IL-8 and the anti-inflammatory cytokine IL-10 were measured by chemiluminescence using the *Immulite 1000* automated chemiluminometer (Siemens Healthcare Diagnostics, Deerfield, IL). All experiments were replicated at least three times using different monocyte donors and plasma units per replicate experiment. Endotoxin and pyrogen-free reagents and labware were used for all experiments. Cell isolation and stimulation were all executed using sterile technique in a tissue-culture hood.

Antigen-Presentation Capacity

Cells from coculture experiments were used to determine antigen-presentation capacity as measured by HLA-DR expression. The percentage of live monocytes expressing the MHC class II molecule HLA-DR after exposure to autologous plasma control, FFP, thawed plasma, SD plasma, or spray-dried SD plasma was measured by flow cytometry using the following stains: Live Dead aqua (Life Technologies), APC anti-human CD14, and fluorescein isothiocyanate (FITC) anti-human human leukocyte antigen (HLA)-DR (Becton Dickinson). COMPtrol antibody capture beads (Spherotech, Lake Forest, IL) or single-stained samples were used to set compensation where appropriate. Positive and negative gates were set using a fluorescence-minus one strategy. Data were acquired with an LSRII flow cytometer (Becton Dickinson) in the flow cytometry core at The Research Institute at Nationwide Children's Hospital. Data were analyzed using FlowJo computer software (TreeStar, Ashland, OR).

Citrate and Ionized Calcium Measurements

Because citrate has been shown to modulate monocyte LPS-induced cytokine production, we evaluated the possibility that differences in cytokine production potential among plasma products could be related to differences in citrate and/or ionized calcium concentrations.[24] We measured citrate concentration in the plasma products by coupled enzyme assay (Sigma-Aldrich, St. Louis, MO) according to manufacturer instructions. Ionized calcium was measured by the clinical laboratory (Siemens 1200 Rapidlab Blood Gas System, Tarrytown, NY). The lower limit of detection for this method is 0.25 mm/L of ionized calcium. To increase ionized calcium concentrations in the plasma aliquots to above this threshold of detection, equal amounts of calcium chloride (7.5 mM) were added to each 1-mL aliquot of plasma product prior to measurement.

Data Analysis

Based on our previous in vitro studies of RBC-induced monocyte immunomodulation, our primary outcome measures included monocyte production of the cytokines TNFα, IL-10, IL1β, and Il-8 in response to LPS stimulation. Secondary outcome measures included monocyte cytokine production in the absence of LPS and monocyte HLA-DR expression. Based on our previous in vitro RBC transfusion models, assuming 80% power and alpha < 0.05, we concluded that six replicates would be necessary to detect a 50% difference in LPS-induced cytokine production using one-way analysis of variance with three pairwise comparisons. Data presented are mean ± SD. Differences between plasma exposure groups and controls were analyzed using analysis of variance with Dunnett or Tukey correction for multiple comparisons, as appropriate. Analyses were performed using Prism6 software (GraphPad Inc., La Jolla, CA). A p value less than 0.05 was considered to be significant throughout.

RESULTS

Monocyte Cytokine Production After Exposure to Plasma Products in the Absence of LPS

Monocyte cytokine production after exposure to plasma products alone are shown in Figure 1. For all plasma exposure groups, production of the anti-inflammatory cytokine, IL-10 was very low across the board with no significant differences related to plasma exposure. Regarding proinflammatory cytokines, monocytes produced significantly more IL-8 following exposure to spray-dried SD plasma compared with autologous plasma controls and other plasma products.

Monocyte LPS-Induced Cytokine Production and HLADR Expression After Exposure to Plasma Products

Monocyte cytokine production after exposure to plasma products and stimulation with LPS are shown in Figure 2. Similar to non–LPS-stimulated monocytes, IL-10 production capacity after exposure to plasma products was not different compared to autologous plasma controls or among plasma products. By contrast, LPS-induced monocyte production of all three inflammatory cytokines, TNFα, IL1β, and IL-8 was significantly higher after coculture with spray-dried SD plasma. Solvent/detergent plasma exposure, however, resulted in lower LPS-induced production of the inflammatory cytokines, TNFα and IL8. Thawed plasma exposure resulted in small, but statistically significant decreases in monocyte HLA-DR production, with HLA-DR remaining normal after exposure to other plasma products (Fig. 3).

Citrate and Ionized Calcium Measurement

Total citrate concentration was lowest in solvent/detergent plasma with no significant differences among spray-dried SD plasma, FFP, or thawed plasma, suggesting that the augmented cytokine production after exposure to spray-dried SD plasma was not due to differences in citrate concentration (Fig. 4A). Additionally, because citrate would be expected to suppress cytokine production, the observed decrease in LPS-induced cytokine production after exposure to SD plasma was not likely due to a lower citrate concentration. As expected, ionized calcium concentrations were highest in SD plasma, again suggesting that lower monocyte cytokine production following SD

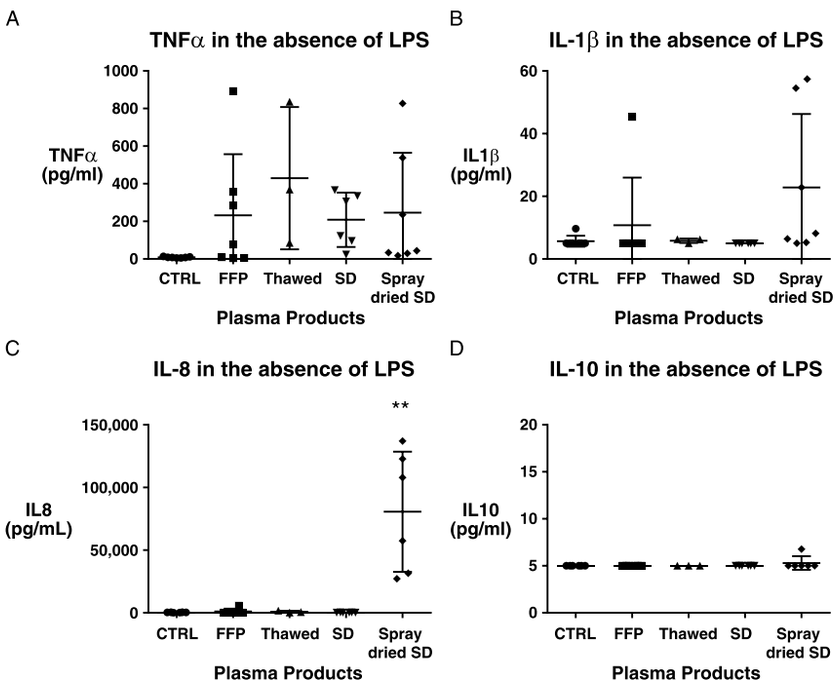

Figure 1. Spray-dried solvent/detergent plasma exposure alone resulted in marked elevation in monocyte IL-8 production. Monocytes were cocultured with 40% by volume FFP, thawed plasma, solvent/detergent plasma (SD) or spray-dried solvent/detergent plasma. Monocyte production of the inflammatory cytokines, TNFα (A), IL1β (B), IL-8 (C) and the anti-inflammatory cytokine, IL-10 (D) were measured. $^{**}p < 0.01$ compared with autologous plasma control. N = 7 replicate experiments.

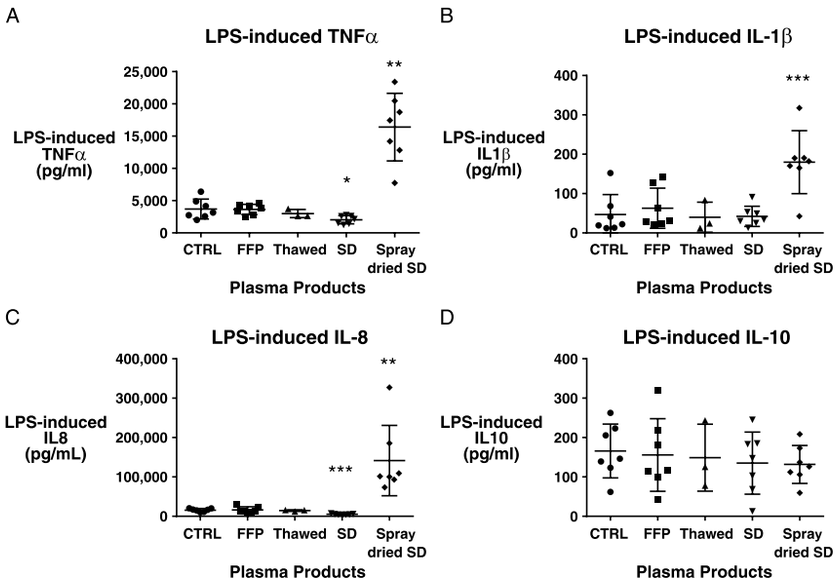

Figure 2. Spray-dried solvent/detergent plasma exposure resulted in elevated monocyte LPS-induced inflammatory cytokine production, while solvent/detergent plasma exposure attenuated inflammatory response. Monocytes were co-cultured with 40% by volume FFP, thawed plasma, solvent/detergent plasma (SD) or spray-dried solvent/detergent plasma. Following LPS stimulation, monocyte production of the inflammatory cytokines, TNFα (A), IL1β (B), IL-8 (C) and the anti-inflammatory cytokine, IL-10 (D) were measured. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with autologous plasma control. N = 7 replicate experiments.

plasma exposure was not due to lack of available calcium in the model (Fig. 4B).

Spray-Dried SD Plasma Reconstitution Solution

To evaluate the possibility that the solution used to reconstitute the spray-dried SD plasma may be immunomodulatory or that transfer of the reconstitution solution introduced pyrogen contamination, experiments were repeated as follows. Monocytes in media were exposed to 40% by volume autologous plasma, 40% by volume autologous plasma plus 20% by volume reconstitution solution, or 40% by volume spray-dried SD plasma. As depicted in Supplemental Figure 2, IL-8 production was again higher after exposure to spray-dried SD plasma compared to autologous plasma controls - both before and after LPS stimulation (see Figure, Supplemental Digital Content 2, http://links.lww.com/TA/B87). Exposure to autologous plasma plus reconstitution solution did not induce IL-8 production.

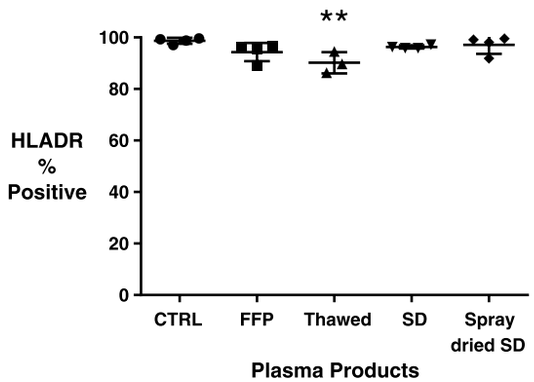

Figure 3. Thawed plasma exposure resulted in small, but statistically significant decrease in monocyte HLA-DR expression. Monocytes were co-cultured with 40% by volume FFP, thawed plasma, solvent/detergent plasma (SD) or spray-dried solvent/detergent plasma. Monocyte HLA-DR expression was measured by flow cytometry. **$p < 0.01$ compared with autologous plasma control. N = 7 replicates experiments.

Pre- Versus Post-Spray-Dried SD Plasma

The SD plasma used to make spray-dried SD plasma is produced using a modification of Octapharma's solvent/detergent process (Kedrion S.p.A, Barga, Lucca, Italy). Therefore, to evaluate the possibility that the augmented cytokine production seen following exposure to spray-dried SD plasma may be due to differences in source SD plasma, we obtained an aliquot of Kedrion solvent/detergent plasma pre-spray drying and additional units of the same lot of solvent/detergent plasma after spray drying. In this set of experiments, spray-dried SD plasma exposure again resulted in significantly augmented monocyte production of the inflammatory cytokines IL-8 and IL-1β (Fig. 5). This was not seen with aliquots of the same SD plasma before the spray-drying process. These findings suggest that augmented cytokine production following spray-dried SD plasma exposure was not likely due to the source of SD plasma.

DISCUSSION

In our study, there was significant variability in immunomodulatory effects of plasma products based on their preparation. While FFP exposure did not significantly alter monocyte cytokine production, exposure to spray-dried SD plasma resulted in significantly higher inflammatory cytokine production, even in the absence of LPS stimulation. By contrast, the lowest cytokine production was observed following exposure to solvent/detergent plasma.

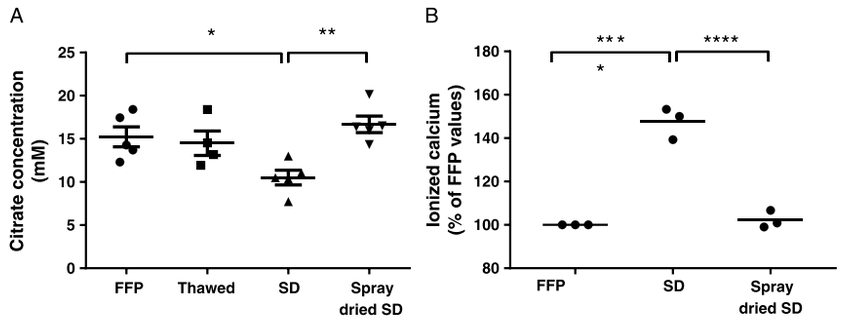

Figure 4. Solvent/detergent plasma contained the lowest concentration of citrate (A) and highest concentration of ionized calcium (B). To bring ionized calcium concentrations in to an optimum measurable range, ionized calcium was measured in plasma products after the addition of 7.5 mM of calcium chloride to each plasma product aliquot. Data are expressed as % of ionized calcium concentrations in FFP. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ compared with FFP. N = 3 replicate experiments.

Our results raise the possibility that spray-dried solvent/detergent plasma exposure may directly activate monocyte IL-8 production and prime monocytes for an augmented inflammatory response to LPS. Interleukin-8 is a neutrophil chemoattractant chemokine released by activated monocytes. Clinically, IL-8 is an important biomarker of inflammation; and elevated circulating IL-8 concentrations are associated with increased mortality across a variety of critically ill and injured patient populations, including sepsis, trauma, and acute respiratory distress syndrome.[25–28] IL-8 production is also associated with blood product transfusion and may be implicated in the pathophysiology of transfusion-related acute lung injury.[29–33] However, clinical relevance of increased IL-8 production secondary to plasma product exposure remains unknown.

The finding of augmented monocyte LPS-induced IL-8 production in the context of alternate blood product processing methods is not unique to our study. Loh et al.[34] demonstrated differential cytokine production from peripheral blood mononuclear cells after exposure to supernatants from Mirasol pathogen reduction technology-treated platelets. Similar to the current study, LPS-induced IL-8 production was significantly increased after PRT-treated platelet supernatant compared to untreated platelet products. These findings support the notion that blood product processing, including some pathogen reduction technologies and lyophilizing methods, may lead to very different immunomodulatory properties which should be evaluated in clinical studies.

One previous study evaluated in vitro immunomodulatory potential of spray-dried SD plasma using an endothelial cell model.[35] In the endothelial cell model, spay-dried SD plasma improved endothelial cell function relative to lactated ringers, performing similarly to FFP. Leukocyte adhesion to endothelial cells was reduced following both FFP and spray-dried SD plasma exposure, though cytokine production was not evaluated.

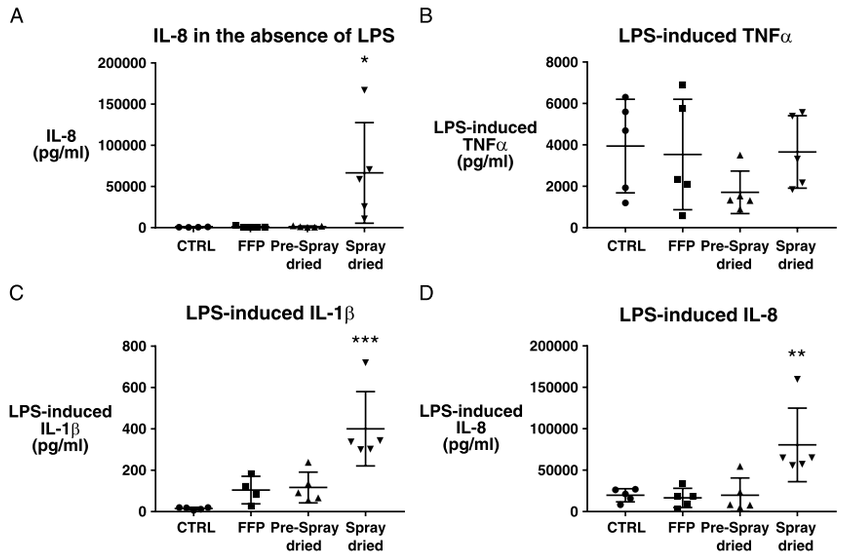

Figure 5. Pre-spray dried plasma was not associated with increase in monocyte cytokine production. Monocytes were cocultured with 40% by volume FFP, pre-spray solvent/detergent plasma, or post-spray dried solvent/detergent plasma from the same lot and manufacturer. Similar to previous experiments, spray-dried plasma exposure resulted in increased monocyte IL-8 production (A) and LPS-induced IL-1β (C) and IL-8 (D) and production. Cytokine production after exposure to pre-spray dried plasma was not significantly different compared to FFP or autologous plasma controls. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with autologous plasma control. N = 5 replicate experiments.

The difference in results compared with the current study is likely due to the two different model systems and cell types used. It is unclear how differential immunomodulatory signaling from innate immune cells and endothelial cells might interact with each other and what their combined effects might be in vivo. We consider this an important area for future investigation.

The finding of decreased cytokine production following exposure to SD plasma is notable. European hemovigilance studies document significant decreases in inflammatory transfusion complications, including TRALI, associated with SD plasma use.[25,36] Likewise, in a post hoc analysis of the PLASMA TV international point prevalence study of plasma transfusion in critically ill children, SD plasma use was independently associated with decreased mortality.[37] These data support a hypothesis that SD plasma use may attenuate plasma-associated immune stimulation in critically ill and injured patients requiring plasma transfusion. On the other hand, the observed reductions in innate immune cell responsiveness following SD plasma exposure in our model may represent potentially harmful immunosuppressive effects. While it is unclear if these effects are severe enough to translate to clinically relevant immune suppression, we view this as an important area for future investigation. More definitive clinical studies are warranted to address these important hypotheses.

Specific mediators of plasma product-induced immunomodulation are unknown. Circulating microvesicles have been implicated as mediators of transfusion-related immunomodulation.[17,38] However, a previous study documented significantly fewer circulating microvesicles in both solvent/detergent and spray-dried solvent/detergent plasma products, suggesting that microvesicles are not likely responsible for the inflammatory effects of spray-dried SD plasma detected in the current study.[21] To evaluate the possibility that our findings may be due to differences in citrate or available ionized calcium across plasma products, we measured citrate concentrations in the plasma units. We were able to demonstrate that the balance of citrate and available calcium was not different between spray-dried SD plasma and FFP suggesting that our findings are not likely an artifact of differential calcium signaling in the model. Ongoing work is needed to identify other potential causal mediators.

Our study has limitations. Because we evaluated only one cell type, we are unable to comment on immunomodulatory effects of plasma products on other immune cells. Potential effects of plasma products on the complex interplay of innate and adaptive immune cells remains an active area of ongoing investigation. Importantly, it is possible that very different immunomodulatory effects may be seen in models which incorporate additional innate immune cell types (ie, neutrophils and natural killer cells) as well as the microenvironment of the inflammatory response. We view these as important next steps. Similarly, our studies were limited to a focused array of immune function readouts. Additional measures of innate immune cell function, including opsonization, phagocytosis, and chemotaxis may be revealing. For instance, an expanded array of immunologic readouts may reveal immunomodulatory effects of FFP units which were not apparent in our analyses. We chose to focus on cytokine production capacity and HLA-DR expression because they are analogous to measures of innate immune function which are associated with adverse clinical outcomes in critically ill and injured patients and can be feasibly measured in multicenter clinical studies.[10,11,14] These readouts, therefore, are easily amenable to translation to the bedside. Because our current study is limited by its in vitro nature, the extent to which our results may translate to clinical effects is unclear. While the in vitro methodology precludes precise comparison to clinical studies, it is notable that the IL-8 production following spray-dried SD plasma exposure is as high or higher than plasma IL-8 concentrations associated with adverse outcomes in critically ill patients, supporting the biologic plausibility that these findings could have clinical relevance. Additionally, in our previous work, older stored red blood cell products suppressed monocyte LPS-induced cytokine production in similar in vitro models and were associated with innate immune suppression in critically ill children, suggesting that our in vitro transfusion models may have a clinical correlate.[14,19] Incorporating assessments of immune function and systemic inflammation into future clinical trials evaluating the safety and efficacy of spray-dried SD plasma products can be done and may shed light on this important question.

In conclusion, we found that monocytes exposed to spray-dried SD plasma exhibited augmented inflammatory cytokine production. By contrast, SD plasma exposure resulted in decreased LPS-induced inflammatory cytokine production. The extent to which these findings may translate to differences in immunomodulatory potential among transfused patients is unclear and is deserving of future study.

AUTHORSHIP

S.S. designed the study, performed experiments and wrote the article. K.C., S.M., S.B., and J.N. performed experiments and revised the article. P.C.S., K.N., and M.W.H. designed the study and revised the article. J.A.M. designed the study, supervised experiments, analyzed and interpreted the data and wrote the article. All authors reviewed and approved the final version of the article.

DISCLOSURE

P.C.S. is a consultant for Octapharma, Cerus, and Entegrion. M.W.H. is on the Scientific Advisory Board of Bristol Meyers Squibb. The other authors declare no potential financial conflicts of interest. This work was supported by the Department of the Navy, Office of Naval Research (to J.A.M.). Spray-dried SD plasma products were supplied by Entegrion. Solvent/detergent plasma products were supplied by Octapharma. J.A.M. is supported by K08HL123925 and The Research Institute at Nationwide Children's Hospital. The aforementioned funders and suppliers played no role in study design, data analysis, manuscript preparation, or decision to publish.

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