Experimental Pre-storage Filtration Removes Antibodies and Decreases Lipid Bioactivity Accumulation in RBC Supernatants Mitigating TRALI in an Animal Model

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Running title: Experimental filtration inhibits priming and TRALI
Key Points

- TRALI may be induced by antibodies to HLA or HNA antigens or lipids which accumulate during storage.
- Pre-storage experimental filtration of RBCs removes HLA and HNA antibodies, decreases lipid priming activity and mitigates TRALI in an animal model.

Abstract

TRALI remains a significant cause of transfusion-related mortality with red cell transfusion. We hypothesize that pre-storage filtration may reduce proinflammatory activity in the RBC supernatant and prevent TRALI. **Methods:** Filters were manufactured for both small volumes and RBC units. Plasma containing antibodies to HLA-A2 or HNA-3a was filtered and immunoglobulins and specific HNA-3a and HLA-2a PMN priming activity were measured. Antibodies to OX27 were added to plasma, and filtration was evaluated in a two-event animal model of TRALI. RBC units from 31 donors known to have antibodies against HLA antigens and from 16 antibody-negative controls were filtered. Furthermore, 4 RBC units were drawn and underwent standard leukoreduction. Immunoglobulins, HLA antibodies, PMN priming activity, and the ability to induce TRALI in an animal model were measured. **Results:** Small volume filtration of plasma removed >96% of IgG, antibodies to HLA-A2 and to HNA-3a, and their respective priming activity, as well as mitigating antibody-mediated *in vivo* TRALI. In RBC units experimental filtration removed antibodies to HLA antigens and inhibited the accumulation of lipid priming activity and lipid-mediated TRALI. We conclude that filtration removes proinflammatory activity, the ability to induce TRALI from RBCs, and may represent a TRALI mitigation step.

**Key Words:** Priming, Neutrophils, RBC Supernatant,
Introduction

Transfusion-related acute lung injury (TRALI) is a significant cause of transfusion-related mortality\textsuperscript{1-4}. Despite the implementation of TRALI mitigation strategies for plasma and platelet concentrates (PCs), which employ either male-only and/or "antibody-negative" donors, there are still significant numbers of TRALI-related deaths, especially linked to the transfusion of red blood cell (RBC) units\textsuperscript{1,2,5,6}. TRALI has been reported to be the result of at least two events: the first pertaining to the clinical condition of the patient and the second to the infusion of antibodies that recognize HLA class I, class II, or granulocyte antigens or the infusion of biologic response modifiers (BRMs), which include both biologically active lipids and soluble CD40 ligand (sCD40L)\textsuperscript{7-16}. Standard pre-storage leukoreduction was reported to have decreased TRALI reactions at one medical center; however, leukoreduction has not affected TRALI incidence in the United States or worldwide\textsuperscript{17,18}. To date there are few RBC mitigation strategies for TRALI.

Standard leukoreduction by filtration removes 3 logs of leukocytes and 2 logs of platelets. Removal of leukocytes and platelets has been shown to reduce: 1) HLA antigen exposure, 2) febrile, non-hemolytic transfusion reactions, 3) levels of cytokines accumulation during routine RBC storage, 4) sCD40L accumulation, and 5) cytomegalovirus exposure\textsuperscript{9,18-21}. The majority of RBC units in most countries is pre-storage leukoreduced either by filtration or buffy coat removal.

TRALI in a susceptible host has been related to the infusion of donor antibodies against the HLA antigens of the recipient, which may reside on either the recipient's leukocytes or endothelial cells, or the infusion of lipids or sCD40L\textsuperscript{22-24}. These antibodies, lipids or, sCD40L activate PMNs allowing for PMN-mediated endothelial damage, capillary leak and acute lung injury (ALI)\textsuperscript{7-9,14,25}. We hypothesize that a pre-storage filter that removes antibodies, leukocytes, and platelets and decreases lipid bioactivity would significantly decrease the presence of donor HLA antibodies, the accumulation of biologically active lipids and sCD40L, and would abrogate TRALI in a two-event animal model.
Materials and Methods

Materials. All chemicals and reagents were purchased from Sigma Chemical Corporation (St Louis, MO) unless otherwise delineated. OX27 antibodies were purchased from AbD Serotec (Raleigh, NC) or Abcam (Cambridge, MA). All buffers were made from stock USP solutions and filtered with Nalgene™ MF75 series disposable sterilization filter units (Fisher Scientific Corp., Pittsburgh, PA)\(^{26}\). ELISA kits for arachidonic acid (AA) or 5-hydroxyeicostetraenoic acid (5-HETE) were purchased from Antibodies-online.com (Atlanta, GA).

Filtration. The experimental filters are based upon proprietary material which primarily removes antibodies. Two types of filters were used in the described experiments: 1) a small volume filter in two sizes for 1-10 ml or 10-50 ml of plasma and 2) filters which are structurally similar to the Pall BPF4 standard leukoreduction filters. These RBC filters contain the identical leukoreduction filter and additional material that adsorbs antibodies and structurally similar proteins and non-specifically binds lipids\(^{27}\). The volume of RBCs that is lost from experimental filtration is 25 ml. With respect to immunoglobulins, the filter removes all four classes (data not shown for IgA and IgE) and is preferentially selective for IgG>IgM. The B, P, and M designations are assigned to different methods of packaging the antibody adsorbent material\(^{27}\).

Experiments employing filters for small volumes. To assess the capability to remove IgG and IgM, five fresh human plasma samples (100 ml) were obtained. 50% by volume was filtered and the other 50% left as an unmodified control (v:v). The amounts of IgG pre- and post-filtration were measured in the clinical laboratory at Children’s Hospital Colorado (Aurora, CO) and confirmed at Pall Corporation.

The ability of the experimental filter to remove immunoglobulins, including HLA-A2 and HNA-3a antibodies, was also measured in the small volume system. Plasma (5 ml) was drawn from three multiparous females: one with antibodies to HNA-3a who was implicated in nine TRALI reactions, another with known antibodies to both HLA class I and class II antigens, and a third with known antibodies to HLA-A2 were divided into equal volumes with 50% filtered and
50% left as an unmodified control. The effects of filtration on PMN priming activity were then completed using matched PMNs (HNA-3a + PMNs for HNA-3a+ plasma and PMNs from homozygous HLA-A2 donors for HLA-A2+ plasma) and sent to clinical laboratories for HLA or HNA antibody measurements. Lastly, to determine if filtration mitigated TRALI in a rat model of antibody-induced TRALI, heat-treated human plasma (2 ml) (56°C for 30 min to destroy human complement and fibrinogen activity) with 50 μg/ml of monoclonal antibodies to OX27, an MHC class I antigen in rats, was divided into equal volumes and was filtered using the smaller (1-10 ml) device. These samples were then used as the second event in a two-event animal model of TRALI to determine if experimental filtration could remove the antibodies to OX27 and mitigate TRALI.

**Collection of RBCs and preparation of RBC supernatants.** Filtration of entire red cell units in AS-5 additive solution from control and donors known to harbor HLA antibodies was similarly investigated. After obtaining informed consent via a protocol approved by the Western Internal Review Board (WIRB), one unit of whole blood (500±50 mL) was collected from two groups of donors. This study was conducted in accordance with the Declaration of Helsinki. The first group consisted of healthy, antibody-negative donors. Specifically, this control group (n=16) consisted of 4 male donors per experimental filter group: B (4), P (4), and M (4), and 4 donors (2 males and 2 females) whose RBC units underwent standard leukoreduction. The second group was comprised of 31 multiparous females who previously tested positive for antibodies to HLA class I antigens, class II antigens, or both by both Luminex™ bead assays with flow cytometry and via the GTI ELISA within the past 4 years (Fig.1). Briefly, whole blood was collected in a CPD with Optisol™ collection bag system (Teruflex, Terumo Corporation, Tokyo, Japan). Plasma was separated from RBCs by centrifugation followed by expression, employing an automated closed system, Compomat G4 ( Fresenius-Kabi, Schweinfurt, Germany) and AS-5 (Optisol) was added to a final hematocrit of 50-60%. The estimated amount of residual plasma per unit was 5-10 ml (6.1±1.0 ml)²⁸. A 10 ml sample was drawn from
each RBC unit prior to and following experimental filtration with the “B”, “P”, or “M” filters, and four control units that underwent standard leukoreduction. The RBC units were stored at 1-6°C, and samples were obtained through sterile couplers. The supernatant was isolated via centrifugation (5000xg for 7 min) followed by a second spin at 12,500xg for 6 min at 4°C to remove contaminating platelets and stored at -80°C.

**Measurement of Antibodies to HLA class I and class II antigens.** Donor antibodies directed against HLA class I antigens, class II antigens, or both were measured using Luminex beads and flow cytometry at two HLA reference laboratories: ClinImmune (Denver, CO) and LABs (Centennial, CO) in a blinded fashion. The mean fluorescence intensity (MFI) data has been included to demonstrate a functional titer/strength of these antibodies. These data were confirmed using identical methodology at Pall Corp. and Bonfils Blood Center.

**Measurement of antibodies to HNA-3a.** Measurement of antibodies to HNA-3a in both pre-filtration and post-filtration plasma samples was performed employing standard techniques at the Granulocyte Laboratory, Blood Center of Southeastern Wisconsin (BCW). The pre-and post-filtration samples were tested in a blinded fashion. The antibody titer was not measured, and the plasma came from a donor who was implicated in nine TRALI reactions.

**Measurement of 2,3-DPG, ATP, pH, IgG, IgM, blood counts, and soluble CD40 ligand (sCD40L) in RBC units.** Prior to and following filtration with the experimental filters (B, P, and M) and standard leukoreduction, samples were drawn and IgG, IgM, 2,3-DPG, and ATP were measured. Measurement of 2,3-DPG, ATP, and pH were completed in the clinical laboratories at BCW and confirmed at Pall Corp. All complete blood counts (CBCs) were performed at BCW, Pall Corp, and Bonfils Blood Center. The IgG and IgM levels were measured at the Children’s Hospital of Colorado and confirmed at Pall Corp. sCD40L was measured via commercial ELISA (R&D Systems, Minneapolis, MN).

**PMN priming assays.** Heparinized whole blood was drawn from healthy donors after obtaining informed consent employing a protocol approved by COMIRB, and the PMNs were
isolated by standard techniques. PMNs (3.75 x 10^5) were then incubated with 10% (v:v) fresh plasma (FP) control, plasma containing antibodies to HLA-A2 or HNA-3a, or the supernatant from days 0 (pre-and post-filtration), 21, or 42 for 5 min at 37°C. The PMNs were then activated with 1 μM fMLF, and the maximal rate of superoxide dismutase inhibitable, superoxide production (O_2^-) was measured. Lipids were isolated from these samples using a 1:1:1 chloroform:methanol:water 0.2% acetic acid extraction. The lipids were solubilized with 1.25% fatty-acid-free Human albumin, and PMNs were incubated with albumin or the lipids for 5 min at 37°C. The PMNs were then activated with 1 μM fMLF, and O_2^- was measured.

**Two-event in vivo model.** Male Sprague Dawley rats (Harlan, Indianapolis, IN) underwent a treatment protocol approved by the Animal Care and Use Committee, UCD. Briefly, rats were injected intraperitoneally (IP) with 2 mg/kg lipopolysaccharide (LPS, *Salmonella enteritides*) incubated for 2 hours, anesthetized with 60 mg/kg pentobarbital and the femoral vessels cannulated. Blood was removed (10 min) equal to 10% of the total blood volume followed by infusion of an identical volume of heat-treated (56°C for 30 min to obviate the effects of fibrinogen and complement) cell-free plasma with 50 μg/ml of OX27±filtration or heat-treated supernatant from day 1 or day 42 RBC units that underwent experimental filtration or standard leukoreduction. These second events were infused at 4 ml/hr followed by 30 mg/kg of Evans Blue Dye (EBD). Six hours later blood (3 ml) was drawn, and the rats were euthanized followed by a bronchoalveolar lavage (BAL). Lung leak was measured as the %EBD in the plasma versus the BAL fluid (BALF).

**Statistics.** All data are presented as the mean ± the standard error of the mean. Statistical differences among groups were measured by independent or paired ANOVAs followed by either a Bonferroni’s or Newman-Keuls *post-hoc* test for multiple comparisons depending upon the equality of variance. Significance was determined at the p<0.05 level.
Results

Filtration of Immunoglobulins and HLA antibodies in small volumes of human plasma. Fresh plasma from five donors was employed to determine IgG removal by experimental filtration. The experimental filter for small volumes (10-50 ml) removed 98±2.1% of the IgG from 50 ml of human plasma (p<.05, n=5). To determine if the experimental filter could remove HLA antibodies, four 1 ml plasma samples from a female donor with antibodies to both HLA class I and class II antigens were employed. The 1-10 ml filter removed 93.5±2.7% of the antibodies to HLA class I antigens and 99.1±0.9% of the HLA class II antibodies; whereas the 10-100 ml filter removed 96.4±1.6% and 99.4±0.4% of the antibodies to HLA class I and class II, respectively.

To document removal of antibodies to HLA class I and HNA antigens plasma samples (3 ml, 50% filtered, v:v) from two female donors known to contain these antibodies were employed. Experimental filtration removed antibodies to HLA-A2 and to HNA-3a from the plasma of two multiparous female donors, as demonstrated by the presence of these antibodies in the unmodified plasma and its absence in the filtered portion measured by two separate reference laboratories. The relative strength/titer, MFI, of the HLA-A2 antibody in the unmodified plasma was 5,615 and 4,930 against HLA-A2 and below the cutoffs of <2,000 for both laboratories. With respect to the HNA-3a antibodies, immunoglobulins against HNA-3a were detectable in the unmodified plasma but could not be detected in the filtered plasma. In addition, because antibodies to HLA-A2 and to HNA-3a prime PMNs from homozygous HLA-A2 positive (HLA-A2⁺) donors and HNA-3a⁺ donors, respectively, the ability of the filters to remove this priming activity was investigated (Fig.2)³³⁻³⁴. Filtration effectively mitigated the priming activity of the HLA-A2⁺ plasma in PMNs from HLA-A2 homozygous donors (Fig.2A). Furthermore, experimental filtration also removed the priming activity from the HNA-3a⁺ plasma in PMNs from HNA3a⁺ donors (Fig.2B).
Filtration of antibodies from plasma inhibits in vivo TRALI. To assess possible in vivo implications of the removal of antibodies from human plasma, 50 μg/ml of OX27 antibody was added to heat-treated human plasma: total of 8 ml divided into 1 ml aliquots with four 1 ml aliquots filtered and four 1 ml aliquots left as unmodified controls, replicated twice. In this two-event model rats were incubated with either saline or LPS for 2 hours (first event) and then infused with the filtered or unfiltered, heat–treated human plasma (second event). In the saline-treated rodents no ALI was observed in any treatment group. However, as compared to the saline-treated animals as well as the LPS-treated rats infused with filtered, heat-treated plasma, the unfiltered plasma containing 50 μg/ml OX27 demonstrated significant ALI (Fig.3). Filtration of the heat-treated plasma containing 50 μg/ml of OX27 mitigated TRALI in this animal model.

Experimental filtration provides leukoreduction, maintains ATP and 2,3 DPG concentrations without affecting pH. As expected, the experimental filters demonstrated adequate leukoreduction by decreasing leukocyte counts by >3 logs and platelet counts <2 logs similar to standard leukoreduction (Table 1). Moreover, experimental filtration did not significantly affect ATP levels throughout the storage interval versus standard leukoreduction on any day of storage. Specifically, the ATP levels were not different pre- and post- experimental filtration and standard leukoreduction on day 0, although experimental filtration modestly increased the ATP concentrations. The ATP concentrations were also not different on day 21 but were significantly decreased on day 42 for each filter group including standard leukoreduction. In addition, the pH range for all units was acceptable although mildly decreased in the experimental groups (Table 1). Lastly the experimental filtration did decrease the 2,3-DPG levels similar to standard leukoreduction (Table 1). The 2,3-DPG levels decreased during storage for all groups by day 42 which was not statistically different from the concentrations in the standard leukoreduction units on day 42.
Pre-storage filtration of RBC units removes and donor antibodies. As compared to the unfiltered samples, filtration removed two logs of IgG and >80% of the IgM in all units tested (Table 1). Of the 31 female donors who previously tested positive for antibodies to HLA class I, class II, or both, 13 donors had HLA antibodies in their pre-filtration samples: 8 had antibodies to HLA class I antigens, 9 had antibodies to HLA class II antigens, and 4 of these donors had antibodies to both HLA class I and Class II antigens (Tables 1 & 2). The strength/titer of these antibodies was calculated as the MFI of the bound Luminex™ beads (Table 2). In addition, the GTI ELISA values were also positive for each of these antibodies (Table 1). Importantly, the antibodies found in the pre-filtration sample were not detected in the filtrate from any of the experimental devices (Table 1).

Pre-storage experimental filtration significantly inhibits the accumulation of PMN priming activity during RBC storage. Experimental filtration significantly decreased the PMN priming activity that accumulates in the supernatant during routine RBC storage vs. standard leukoreduction (Fig.4A). There was no difference in priming activity in day 0 RBC supernatants, either pre- or post-filtration in any of the experimental filtration RBC supernatants vs. standard leukoreduction. At day 21 and day 42 the standard leukoreduced control supernatants exhibited significantly greater priming activity than any of the supernatants from the experimentally filtered RBCs (Fig.4A) (p<.05). There was significant priming activity on day 42 versus day 0 in the RBC supernatants, respectively, in each of the experimentally filtered units (p<.05).

Lipid priming activity was measured on samples from all three experimental filters and demonstrated significantly less lipid priming activity compared to standard leukoreduction (Fig.4B). In addition, there was a significant decrease in 5-HETE accumulation but not in AA compared to standard leukoreduction on day 42 with the pre- and post-experimental filtration having no effect on lipid concentrations on day 42 from standard leukoreduction: AA, 1,200±250
ng/ml vs. experimental filtration: 1,321±119 g/ml (n=20) and 5-HETE, 38±11 ng/ml vs. 19±5 ng/ml* (n=14, *=p<.05 vs. 5-HETE standard leukoreduction).

Experimental filtration inhibits stored RBC induced TRALI in a two-event in vivo model. To determine if the decreased post-filtration priming activity in the RBC supernatants could serve as the second event in a two-event model of ALI, heat-treated RBC supernatants were used as the second event in a well described two-event in vivo model of TRALI7. In congruence with previous data, no ALI was demonstrated in saline-treated rodents for any second event. However, the supernatants from day 42 LR-RBCs (standard leukoreduction) caused ALI as the second event in LPS-treated rats (Fig.5). Importantly, none of the supernatants from day 42, or day 0, RBCs that underwent experimental filtration caused similar ALI in LPS-treated rats (Fig.5).
Discussion

The presented data demonstrated that the experimental filters developed for small volume filtration removed >96% of IgG, 93% of antibodies to HLA class I antigens, and 99% of antibodies to HLA class II antigens. The small volume filters also took away the *in vitro* priming activity of two antibodies implicated in TRALI, e.g. antibodies to HNA-3a and HLA-A2\textsuperscript{33,34}. In addition, such removal of antibodies inhibited ALI in a two-event, *in vivo* animal model of TRALI, because by removal of antibodies to OX27 resulted in a second event that did not induce TRALI in LPS pre-treated rats. In contrast, the unfiltered heat-treated plasma that contained antibodies to OX27 did elicit TRALI as the second event in LPS-treated rats.

When the experimental filters were used in the preparation of RBC units in comparison to standard leukoreduction, experimental filtration decreased the amount of 2,3-DPG by day 42 of storage; however, there was not a difference between experimentally filtered units and the standard leukoreduced controls. Surprisingly, the ATP levels modestly increased post-experimental filtration, but the ATP levels at day 42, the end of storage for AS-5 stored units, were not different from the leukoreduced controls. Pre-storage filtration with the experimental filters decreased leukocyte counts by 3.5 logs, platelets by 2 logs, and sCD40L by >90%, identical to standard leukoreduction\textsuperscript{9}. Moreover, experimental filtration decreased the accumulation of pro-inflammatory priming activity in the RBC supernatant, specifically lipid priming activity, as compared to RBC units that underwent standard leukoreduction. Measurement of two lipid moieties, specifically AA and 5-HETE, demonstrated no change in AA and a 50% decrease in 5-HETE. This decrease in lipid pro-inflammatory priming activity mitigated TRALI in a two-event animal model, when day 42 supernatants from experimentally filtered RBC units were used as the second event versus day 42 supernatants from leukoreduced RBC units. Lastly, the amount of residual plasma may differ in RBC units and the amount of antibody adsorbent material in the B, P, and M filters would remove >96% of all IgG from 50 ml of plasma; therefore, these experimental filters could be employed for RBC units with
larger amounts of residual plasma. However, these filters are not designed for whole blood and would likely require more antibody adsorbent material.

Pre-storage leukoreduction is done differently depending upon the locale either by buffy coat removal or by filtration. There has been a recent controversy as to whether lipids accumulate in RBC units due to plasma contamination. However, being that these leukoreduction techniques are not synonymous, the techniques used by these investigators to identify lipids and detect PMN priming is dissimilar to previous studies, and the amount of contaminating plasma is small (5-10 ml, mean of 6±1 ml), direct examination of leukoreduction methodology and RBC storage needs to be completed using identical donors at the same institution.

The in vivo model previously demonstrated that EBD leak was synonymous with ALI employing 5 other measurements including histology, increased protein concentration and CINC-1 in the bronchoalveolar lavage fluid, MPO in the lung, and histological examination of the lung tissue. In the reported data, lung leak was used for it is the most consistent marker of ALI in this model. With respect to OX27, concentration-response data was completed previously in rats as a second event in this in vivo model. OX27 concentrations of <50 μg, e.g. 25 μg (0.075 mg/kg), did not cause ALI in this model, and the filter for small volumes employed removes 93% of all IgG regardless of the species of origin, human or murine.

Experimental filtration decreased 1) the accumulation of PMN priming activity in the day 42 RBC supernatant, 2) the lipid priming activity in the day 42 RBC supernatant, and 3) the concentration of 5-HETE in comparison to leukoreduced control RBCs. These filters were constructed to remove immunoglobulins and do passively bind lipophilic compounds and a number of enzymes and other cofactors. The decrease in 5-HETE accumulation without any decrease in arachidonic acid, the most common precursor, is likely due to removal of the enzyme(s) responsible for its conversion. We have previously reported that both 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP) are present in RBCs units,
and their activity likely results in the accumulation of 5-HETE\textsuperscript{38}. In addition, there is 12-17% sequence homology between Human IgG and FLAP or Human IgG and 5-LO, which may account for clearance of one of these enzymes by experimental filtration\textsuperscript{39}. The PMN priming activity that accumulates in the supernatant during LR-RBC storage is almost totally comprised of lipophilic compounds which were significantly decreased by experimental filtration\textsuperscript{9,21}.

Experimental filtration for RBC units represents a possible method for TRALI mitigation via antibody removal, which to date has not been implemented in most blood centers. Washing of RBC units removes pro-inflammatory activity, including both antibodies and lipids, and ALI may be mitigated in high risk populations by washing of cellular components\textsuperscript{40-42}. TRALI secondary to RBC infusion results in a number of reported fatalities and that number has likely increased with antibody-negative plasma transfusion strategies\textsuperscript{1-3,5,43,44}. Moreover, removal of the lipophilic compounds may also diminish the clinical effects of stored RBCs especially in the critically ill and in the injured. Although never associated with RBC storage age, TRALI did significantly correlate with PC age and, more importantly, with the amount of pro-inflammatory lipid priming activity present in the implicated PCs\textsuperscript{14}. Unfortunately, many investigators equate pro-inflammatory bioactivity with storage age because the bioactivity reaches a relative maximum at product outdate. However, TRALI was associated with stored PCs that contained increased bioactivity as compared to similar PCs with the same storage age, which did not cause adverse events when transfused\textsuperscript{14}. A recent prospective study of TRALI did not demonstrate an association with lysophosphatidylcholines (lyso-PCs); however, lyso-PCs do not accumulate during storage of leukoreduced RBCs and there were enough LR-RBC units to mar these analyses\textsuperscript{21,44}. Furthermore, there are a number of clinical trials investigating the role of stored versus fresh RBC transfusion in the critically ill in whom TRALI may be as common as 5-8% of transfused patients\textsuperscript{45,46}. In a series of injured patients controlled for the number of units transfused, the older units were associated with the development of multiple organ failure, which invariably is initiated by ALI and is a significant cause of patient morbidity and mortality\textsuperscript{47}. One
may hypothesize that such filtration with removal of antibodies, leukocytes and sCD40L may make transfusions safer with diminution of the pro-inflammatory activity of RBC units.

Further work is certainly required including safety and efficacy of the clinical effects of experimental filtration including the introduction of organic compounds, which filtration imparts, and possible adverse events as well as RBC survival studies in vivo. These filters are designed for pre-storage leukoreduction of RBC units, and have nothing to do with bedside filtration or any of the adverse reactions that such a process may impart. One may question, why employ pre-storage filtration if lipids accumulate during routine storage reaching a relative maximum at outdate. The observed diminution of the lipid bioactivity, specifically 5-HETE, was not anticipated and may be due to binding of the enzymes required for its generation from AA. Lung inflammation has also been linked to the RBCs themselves, especially those without the Duffy antigen, and such filtration would not mitigate such effects related to stored RBCs. In conclusion, the presented data describe a method for TRALI mitigation for RBCs that may ultimately make transfusions safer, especially for the critically ill and traumatically injured.
Acknowledgements

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Authorship Contributions

CCS designed the bulk of the experiments and wrote the manuscript. MRK performed priming and all of the animal work and contributed to writing of this manuscript. SYK performed priming experiments and contributed to the writing of this manuscript. ML was responsible for all data sent to the reference laboratories and its compilation. FBW ensured that all RBC components were manufactured properly and contributed to the writing of the manuscript. KJL helped with the experimental design, organized the roles of Bonfils Blood Center and contributed to the writing of the manuscript. BM and LC were vital to the manufacture of all the experimental filters performed all of the experiments completed by Pall Corporation and compiled all data. SS-C contributed significantly to the Experimental Design, designed and supervised experimental filter manufacture and helped to write the manuscript.

Conflict of Interest Disclosure

All experiments were paid for by a grant from Pall Corporation. BM, LC, and SS-C were paid employees of Pall Corporation. S-SC is currently a paid employee of Haemonetics Corporation.
CCS received a one-time honorarium in 2009 for a presentation at Pall Corporation. The other authors have no conflicts of interest.
References


Table 1: WBC, Plt, Hct, IgG, IgM, HLA I & II, 2,3-DPG, ATP, and pH in RBC units which underwent experimental filtration or standard leukoreduction.

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<td>18.28±0.25</td>
<td>17.42±0.21</td>
</tr>
<tr>
<td>Post</td>
<td>17.11±1.92</td>
<td>18.14±0.22</td>
<td>18.18±0.30</td>
</tr>
<tr>
<td>Post</td>
<td>18.89±0.30</td>
<td>18.03±0.43</td>
<td>18.03±0.33</td>
</tr>
<tr>
<td>IgG (mg/dL)</td>
<td>Post</td>
<td>121.9±8.1</td>
<td>167.1±31.5</td>
</tr>
<tr>
<td>Post</td>
<td>12.4±11.9*</td>
<td>2.64±2.3*</td>
<td>2.23±0.9*</td>
</tr>
<tr>
<td>IgM (mg/dL)</td>
<td>Post</td>
<td>8.3±1.1</td>
<td>8.69±1.2</td>
</tr>
<tr>
<td>MFI</td>
<td>HLA I</td>
<td>5568.06±938.78</td>
<td>3037.52±202.22</td>
</tr>
<tr>
<td>HLA II</td>
<td>Pre</td>
<td>&lt;2000</td>
<td>&lt;2000</td>
</tr>
<tr>
<td>HLA II</td>
<td>Post</td>
<td>2837.67±163.46</td>
<td>3163.15±202.09</td>
</tr>
<tr>
<td>HLA II</td>
<td>Post</td>
<td>&lt;2000</td>
<td>&lt;2000</td>
</tr>
<tr>
<td>HLA I (# of donors)</td>
<td>Post</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>HLA II (# of donors)</td>
<td>Post</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2,3-DPG (μmol/gHb)</td>
<td>Pre</td>
<td>253.8±12.4</td>
<td>275.0±12.8</td>
</tr>
<tr>
<td>ATP (μmol/gHb)</td>
<td>Pre</td>
<td>4.4±0.3</td>
<td>4.8±0.1</td>
</tr>
<tr>
<td>HLA I (# of donors)</td>
<td>Pre</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>HLA II (# of donors)</td>
<td>Pre</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>pH Range (D42/D0)</td>
<td>Post</td>
<td>6.5/7.3</td>
<td>6.6/7.4</td>
</tr>
</tbody>
</table>

* signifies p<0.05 from day (D) 0 pre-experimental filtration supernatant (Pre) or standard leukoreduction. ** indicates that antibodies to both HLA class I and class II were present in the same donor.
Table 2: HLA Class I and Class II Antibodies Found in Donors used for Experimental Filtration

<table>
<thead>
<tr>
<th>Donor # (Filter Type)</th>
<th>HLA Class I</th>
<th>HLA Class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFI (±SE)</td>
<td>3784.26 (±405.07) [2044.73-10,653.02]</td>
<td>3540.38 (±265.75) [2286.94-7938.03]</td>
</tr>
<tr>
<td>12 (P)</td>
<td></td>
<td>DQ3, DQ7, DQ8, DQ9</td>
</tr>
<tr>
<td>15 (B)</td>
<td>B35</td>
<td></td>
</tr>
<tr>
<td>19 (M)</td>
<td></td>
<td>DR4</td>
</tr>
<tr>
<td>21 (M)</td>
<td>Cw5, Cw15, Cw17</td>
<td>DR13</td>
</tr>
<tr>
<td>24 (M)</td>
<td>B7, B27, B40, B48, B60, B61, B81</td>
<td>DR1, DR7, DR103, DR51</td>
</tr>
<tr>
<td>25 (M)</td>
<td>Cw6, Cw15</td>
<td>DR7</td>
</tr>
<tr>
<td>29 (B)</td>
<td></td>
<td>DR4, DR7, DQ7, DQ9</td>
</tr>
<tr>
<td>30 (B)</td>
<td></td>
<td>DQ1, DQ5, DQ6</td>
</tr>
<tr>
<td>37 (P)</td>
<td>B12, B21, B40, B41, B44, B45, B49, B50, B60, B61</td>
<td>DR3, DR5, DR6, DR8, DR11, DR12, DR13, DR14, DR17, DR18</td>
</tr>
<tr>
<td>39 (P)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 (P)</td>
<td>B35</td>
<td></td>
</tr>
<tr>
<td>43 (M)</td>
<td>B48, B60, B81</td>
<td>DR4, DR7, DR9</td>
</tr>
<tr>
<td>48 (B)</td>
<td>A10, A19, A25, A26, A29, A30, A31, A32, A33, A34, A43, A66, A74</td>
<td></td>
</tr>
</tbody>
</table>

CREG groups: A10 (A25, A26, A34, A66); A19 (A29, A30, A31); B12 (B44, B45); B21 (B49, B50); B40 (B60, B61); DQ1 (DQ5, DQ6); DQ3 (DQ7, DQ8, DQ9); DR3 (DR17, DR18); DR5 (DR11, DR12); DR6 (DR13, DR14)
Figure Legends

Figure 1. Filtration algorithm of HLA antibody positive donors and antibody-negative controls. The algorithm depicts the experimental design of the donors employed for manufacture of RBC units that underwent experimental filtration using filters B, P or M or standard leukoreduction (Pall BPF4 leukoreduction filter). Please note that there are 4 antibody negative male controls per experimental filter and 4 antibody negative donors, 2 male and 2 female for the BPF4 leukoreduction filter.

Figure 2. Experimental filtration mitigates the priming activity of Human antibodies against HLA-A2 and HNA-3a. Panel A: Priming of the fMLF-activated, superoxide dismutase inhibitable, respiratory burst (nmol O$_2^\cdot$/min) in PMNs from HLA-A2 homozygotes primed with fresh plasma, unmodified plasma with antibodies to HLA-A2 and filtered plasma with antibodies to HLA-A2. Experimental filtration mitigated the priming activity which was not different from the fresh plasma-treated controls. Panel B: Priming of the fMLF-activated, superoxide dismutase inhibitable, respiratory burst in HNA-3a$^+$ PMNs primed with fresh plasma, unmodified plasma which contains antibodies to HNA-3a, and experimentally filtered plasma that contains antibodies to HNA-3a. Experimental filtration mitigated the HNA-3a priming activity. (*=p<.05 vs. all groups, n=5).

Figure 3. Experimental filtration mitigates ALI in plasma that contains MHC class I antibodies to OX27. The figure illustrates Evans blue dye (EBD) leak, a measure of ALI, as a function of treatment group. Saline treated rats (first event) did not demonstrate ALI irrespective of the second event: heat treated (56°C for 30 min) fresh plasma (FP), fresh plasma with OX27 (FP+OX27), filtered fresh plasma (F-FP) or filtered fresh plasma + OX27 (FP+OX27). In comparison, endotoxin (LPS) pretreatment did evidence ALI with plasma that contained OX27 (LPS/FP+OX27) as the second event but no ALI was evident in LPS-treated rats infused with
plasma (LPS/FP). Experimental filtration of the plasma with OX27 mitigated ALI (LPS/FP+OX27) and there was no ALI produced by filtered fresh plasma (LPS/FP). *=p<.05 versus all groups, n=5.

Figure 4. Experimental filtration mitigates the accumulation of PMN priming activity and lipid priming activity in the supernatant of RBC units during routine storage. Priming of the fMLF-activated, superoxide dismutase inhibitable respiratory burst (nmol O$_2^-$/min) is depicted as a function of experimental filtration groups and days of routine storage (Panel A). The standard leukoreduced controls (standard LR filter) demonstrate the accumulation of PMN priming activity as a function of storage with significant activity reached by day 21 with a relative maximum on day 42 of storage (*=p<.05 vs. day 1 supernatant). In the three experimental filters (B, P, and M) there is only an increase in priming activity in the RBC supernatants on day 42 vs. day 1 (*=p<.05 vs. day 1 supernatant). The priming activity from supernatants from RBC units that underwent standard leukoreduction was significant from the experimental filtration groups on both day 21 and day 42 (†=p<.05 vs. day 21 and day 42 supernatants from experimentally filtered RBC units; n≥10 for each group). Experimental filtration inhibited the accumulation of lipid priming activity in the supernatant of RBC units during storage (Panel B) depicted as the maximal rate of superoxide anion production to fMLF (nmol O$_2^-$/min) as a function of treatment group. The lipid extracts from the supernatant of the RBCs which underwent standard leukoreduction or experimental filtration were not different from albumin-treated controls. However, extracts from the supernatant of RBCs that underwent standard leukoreduction demonstrated increased priming activity as compared to albumin treated controls, extracts from day 0 and day 42 supernatants from experimentally filtered RBCs *=p<.05 versus albumin controls and lipid extracts from day 1 supernatants, †=p<.05 versus day 42 extracts or the supernatants of experimentally filtered RBCs; n=15). Lastly, the extracts from the supernatants of experimentally filtered RBC units showed greater priming activity vs. the
albumin-treated controls and the extracts from the day 0 experimentally filtered extracts (*=p<.05 versus albumin controls and lipid extracts from day 1 supernatants).

Figure 5. Experimental filtration inhibits ALI induced by the supernatant from stored, day 42 LR-RBCs. EBD leak is depicted as a function of treatment group. Normal saline (NS) treatment of rats as the first event did not result in ALI regardless of the second event: NS, or the heat-treated supernatants from day 1 or day 42 plasma from units that underwent experimental filtration of standard leukoreduction. In LPS-treated rats (first event) NS did not cause ALI; however, the heat-treated supernatants from day 42 RBCs which underwent standard leukoreduction induced significant EBD leak/ALI. Conversely, none of the heat-treated supernatants from the experimentally filtered units on day 42 induced significant EBD leak/ALI. (*=p<.05 vs. LPS/NS and NS/NS controls, n=5 for each group).
Figure 1

47 donors

31 HLA Females

12 HLA Males

4 HLA Controls

Experimental Filters

Standard BPF4 Leukoreduction Filter

B (11)  P (10)  M (10)  B (4)  P (4)  M (4)  M (2)  F (2)
Figure 2

A.

B.

nmoL O₂/min

FP Plasma HLA-A₂ Filtered Plasma

FP Plasma HNA-3a Filtered Plasma

*
Figure 3
Figure 4

A.

B.
Experimental pre-storage filtration removes antibodies and decreases lipid bioactivity accumulation in RBC supernatants mitigating TRALI in an animal model

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