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Effect of leukoreduction on inflammation in critically ill dogs receiving red blood cell transfusions: A randomized blinded controlled clinical trial

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Abstract

Background: Prestorage leukoreduction of red blood cell (RBC) bags prevents accumulation of pro-inflammatory mediators and experimentally attenuates post-transfusion inflammation in healthy dogs. However, the effect of leukoreduction on post-transfusion inflammation in critically ill dogs is unclear.

Hypothesis: Dogs transfused with leukoreduced (LR) RBC will have lower concentrations of leukocytes, interleukin (IL)-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and C-reactive protein (CRP) within 24 hours of post-transfusion compared to dogs transfused with nonleukoreduced (NLR) RBC.

Animals: Sixty-one RBC-transfused dogs (LR = 34, NLR = 27).

Methods: Randomized, blinded, controlled preliminary clinical trial. Blood bag processing was randomized to create identically appearing LR and NLR bags. Group allocation occurred with transfusion of the oldest compatible RBC bag. Blood samples were collected pretransfusion and at 8 and 24 hours post-transfusion for leukocyte count, IL-6, IL-8, MCP-1, and CRP. Data were analyzed on an intention-to-treat basis using linear mixed effects models. Significance was set at $P < .05$.

Results: No significant differences were found between groups in concentrations of leukocytes ($P = .93$), IL-6 ($P = .99$), IL-8 ($P = .75$), MCP-1 ($P = .69$), or CRP ($P = .18$) over time. Eleven LR dogs (32%) and 4 NLR dogs (15%) were euthanized in the hospital ($P = .14$). No natural deaths occurred.

Conclusions and Clinical Importance: No differences in inflammation biomarker concentrations were detected over time between dogs transfused with LR or NLR RBC, but heterogeneity likely hampered the ability to detect a difference with this sample size. The novel randomization and enrollment protocol was successfully implemented across 2 participating institutions and will be easily scaled up for a future multicenter clinical trial.

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1 | INTRODUCTION

Red blood cell (RBC) transfusions are lifesaving in critically ill anemic and bleeding dogs, but they are not without risk. Allogenic RBC transfusions in critically ill people and autologous RBC transfusions in healthy dogs induce an acute inflammatory response, evidenced by post-transfusion increases in inflammation biomarkers including leukocyte count, C-reactive protein (CRP), interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1).[1-5] Transfusion-related inflammation can manifest clinically as acute transfusion reactions such as febrile nonhemolytic transfusion reactions (FNHTR).[6-8] Although FNHTR are not directly life-threatening, the physiologic mechanisms that generate fever can negatively impact patient morbidity by increasing metabolic demand and oxygen consumption.[9] This situation can be particularly deleterious when oxygen delivery is compromised by anemia or hypovolemia.

Post-transfusion inflammation may result from administration of pro-inflammatory mediators that accumulate in RBC products during storage.[10] Specifically, IL-1β, IL-6, IL-8, and tumor necrosis factor-α accumulate in human blood products over time.[4,11,12] Similarly, IL-8 concentration increases in canine RBC products, with an estimated peak between 20 and 30 days of storage.[13-15] Cytokines are derived from leukocytes and platelets within blood products, which can be removed before storage using a leukoreduction filter. In human medicine, leukoreduction has resulted in significant decreases in FNHTR, and is used routinely in some countries.[16-18]

Leukoreduction prevents accumulation of several pro-inflammatory mediators including IL-8 in canine stored RBC, and therefore may attenuate the acute inflammatory response after transfusion of older stored blood.[13-15,19,20] A preclinical trial in healthy research dogs found leukoreduction significantly decreased the acute inflammatory response seen after transfusion of 21-day-old RBC compared with similarly aged nonleukoreduced (NLR) RBC.[5] This effect has not yet been demonstrated in more heterogenous populations of critically ill dogs. Two recently published blinded randomized clinical trials compared biomarkers of inflammation[21] or incidence of acute transfusion reactions[22] between critically ill dogs administered either LR or NLR RBC, and both trials found no difference between groups for either outcome. Both trials included relatively large proportions (57%[21] and 47%[22]) of dogs with immune-mediated diseases for which immunosuppressive medications were administered. Additionally, both trials administered blood that was stored for a mean of ≤16 days. Use of relatively fresh RBC attenuates the difference in pro-inflammatory mediators that accumulate over time between LR and NLR bags. Additionally, immunosuppressive treatment may attenuate the post-transfusion inflammatory response, obscuring any between-group difference in post-transfusion inflammation that may have been present in nonimmunosuppressed dogs. Therefore, it remains unclear if leukoreduction mitigates post-transfusion inflammation in critically ill dogs that are not receiving immunosuppressants.

The aim of our randomized, blinded, controlled clinical trial was to assess the effect of leukoreduction on the post-transfusion acute inflammatory response in nonimmunosuppressed critically ill dogs. We hypothesized that dogs receiving LR RBC would have lower concentrations of leukocytes, IL-6, IL-8, MCP-1, and CRP within 24 hours post-transfusion compared to dogs receiving NLR RBC. Our secondary aim was to generate feasibility and outcome data to be used in planning a larger multicenter trial assessing clinical endpoints.

2 | MATERIALS AND METHODS

This study was led by investigators at Murdoch University (MU) with the University of Queensland (UQ) participating as a secondary study site. The study protocol was approved by MU (R2883/16) and UQ (SVS/568/17) Institutional Animal Ethics Committees.

2.1 | Randomization and blinding

Online random group allocation software (http://www.randomization.com), accessible by both sites, was used to randomize all blood donations collected during the study period as LR or NLR. A blood bank technician at each site who was not involved with blood product administration or care of enrolled dogs was responsible for blood collection and processing. Each RBC bag was assigned a unique code identifier, which was recorded in a password-protected database. The technician labeled bags either A or B, according to group allocation. Because both LR and NLR bags were otherwise identical, clinical staff and investigators were blinded to group allocations. Labeled RBC bags were stored together at 2°C to 6°C in a dedicated blood refrigerator, in order of expiratory date, and freely available for clinical use for a maximum of 42 days. All dogs requiring a blood transfusion received the next available compatible RBC bag, regardless of study eligibility. There was no additional randomization at the study participant level. Unblinding occurred after statistical analysis.

2.2 | Blood collection and processing

Whole blood collection was performed routinely from community donors at MU and from a teaching colony of dogs at UQ.[23] All donors

were blood typed for dog erythrocyte antigen (DEA) 1 using a commercial immunochromatographic testing kit (Quick Test DEA 1, Alvedia, Lemonest, France) before blood collection, and infectious disease testing followed recommendations in published guidelines,[24] where risk of infection was assessed based on geographical location. A total volume of 450 ± 45 mL of fresh whole blood was collected from each donor. Additionally at MU only, blood was collected from DEA 1-typed ex-racing greyhounds as previously described.[14] This blood collection protocol was approved by the MU Institutional Animal Ethics Committee (NC3032/18).

At both study sites, blood bags (MRE system [LR], Macopharma, Rue Lorthiois, France) randomized into the LR group were held at room temperature for up to 2 hours, according to the manufacturer's instructions, to allow the blood to come to ambient temperature for filtration. Each bag then was gently mixed by hand, and the seal was broken to allow the fresh whole blood to flow by gravity through an integrally attached leukoreduction filter (Leucoflex LXT filter, Macopharma) into a satellite bag. Both LR and NLR (FQE system [NLR], Macopharma) whole blood bags then were processed identically as follows. Briefly, bags were centrifuged at 4000g for 10 minutes at 4°C. After centrifugation, plasma was extracted into a satellite bag and removed. Finally, 100 mL of the RBC preservative, saline, adenine, glucose, and mannitol from another integrally attached bag, was added to the bag of RBC and gently mixed by hand. Bags were labeled with their group allocation (A or B) and blood type (DEA 1 positive or negative). They were gently mixed weekly and otherwise were stored vertically in a dedicated blood refrigerator for a maximum of 37 days.

2.3 | Case selection and enrollment

All dogs that were prescribed a RBC transfusion were eligible for inclusion in the study. Exclusion criteria included previous enrollment in this study, administration of immunosuppressive drugs within the previous 24 hours or anticipated in the next 24 hours, delivery of a RBC bolus defined as a rate ≥20 mL/kg/h at any time during the transfusion, or if the enrolling clinician anticipated the patient had a very high likelihood of death, euthanasia, or discharge before the final blood sampling time point (see the Sampling of blood section). Immunosuppressive drugs were defined as corticosteroids administered at immunosuppressive doses (eg, prednisolone at ≥2 mg/kg/d or equivalent), or any dose of cyclosporine, azathioprine, mycophenolate, or leflunomide.

Informed owner consent for study enrollment was obtained in-person or during a telephone conversation by a hospital clinician. Recipient dogs were blood-typed before transfusion and allocated to the LR or NLR group by selection of the oldest type-compatible RBC bag in the blood bank. If dogs were not blood-typed, they were allocated to the LR or NLR group by selection of the oldest DEA 1 negative RBC bag in the blood bank. Per hospital policy, cross-matching was recommended in dogs that had received blood products before 3 days before enrollment. The clinician prescribing the RBC transfusion recorded patient allocation group, A or B, on the standardized

case report form. Once a patient had been allocated to a group, all RBC bags administered within the 24-hour study period were selected from that same group. Dose and rate of delivery of RBC transfusion was at the discretion of the treating clinician and was based on clinical conditions and requirements of each patient.

2.4 | Sampling of blood

Before starting the RBC transfusion, a 5 mL sample was aseptically collected from all RBC bags administered within the 24-hour study period. These samples were centrifuged, and supernatant stored at −80°C for later analysis of canine-specific IL-8. Because IL-8 has been shown to accumulate in canine NLR but not LR RBC bags over time,[13-15] this biomarker was chosen to act as a marker for development of a storage lesion that was expected to be different between NLR and LR RBC bags.

Blood samples were collected from the transfusion recipient at 3 time points, corresponding to baseline or 0 hours, defined as within 1 hour before the start of transfusion, and at 8 hours and 24 hours after the start of transfusion. If >1 transfusion was administered, all time points continued to be defined from the start of the first transfusion. We selected our inflammation biomarkers and 2 post-transfusion time points of 8 hours and 24 hours based on 2 studies assessing post-transfusion inflammation in healthy dogs, which reported peak concentrations of IL-6, IL-8, and MCP-1 at 6 hours, leukocytes at 12 hours, and CRP at 24 hours post-transfusion.[1,5] We felt our chosen time points would best detect peak changes from baseline within the first 24 hours post-transfusion, without excessive sampling of blood.

Blood anticoagulated with EDTA at each time point was used to perform an automated CBC within 24 hours of collection, using 1 of 3 hematology analyzers (XT-2000i, Sysmex, Kobe, Japan; Cell-Dyn 3700, Abbott, Illinois, USA; VetScan HM5, Abaxis, California). Blood samples in lithium heparin tubes and serum separator tubes taken at each time point immediately were centrifuged at 7000g for 10 minutes at 4°C, and separated into aliquots that were stored at −80°C for later batch analysis of plasma IL-6, IL-8, MCP-1, and serum CRP.

2.5 | Monitoring and data collection

Rectal temperature, heart rate, respiratory rate, indirect arterial blood pressure, and rate of transfusion administration were recorded on a standardized case report form (Supporting information, Appendix 1) at the start of transfusion, and then at 15, 30, and 60 minutes later, with subsequent recordings at 60-minute intervals until the transfusion was complete. These variables also were recorded at sampling time points 0, 8, and 24 hours. Monitoring and data collection were performed by clinical staff and final year veterinary students. Any observed signs consistent with a transfusion reaction (including urticaria, facial edema, acute hypotension, pigmenturia, tachypnea, ptyalism, regurgitation, or vomiting) were reviewed by the clinician on

duty, who then decided if cessation of transfusion or treatment or both was indicated.[25] All adverse events and interventions were recorded on the patient's case report form. The ages of the transfused RBC bags in days for each dog were calculated and recorded on a patient demographic spreadsheet after study completion.

2.6 | Biomarker analysis

At the completion of patient enrollment, all frozen serum and plasma samples were shipped overnight on dry ice from UQ to MU for sample analysis. All analyses were performed using first-thaw plasma aliquots and test kits validated in dogs by the manufacturer. Canine-specific IL-6, IL-8, and MCP-1 were measured in duplicate in patient plasma samples according to the manufacturers' guidelines (Milliplex MAP Kit, EMD Millipore Corp, Billerica, Massachusetts) using a multiplexed magnetic bead biomarker analyzer (MAGPIX xMAP, Luminex Corp, Austin, Texas; Bio-Plex MAGPIX Multiplex Reader, Bio-Rad Laboratories Pty Ltd, Hercules, California). Canine-specific IL-8 concentrations in RBC supernatant were measured in duplicate using a commercial sandwich ELISA (Canine IL8 ELISA Kit, Abcam Australia Pty Ltd, VIC, Australia) according to the manufacturer's guidelines. Serum canine CRP was measured using a high throughput biochemical analyzer (AU 480 Chemistry Analyzer, Beckman Coulter Australia Pty Ltd, NSW, Australia) at a commercial laboratory.

2.7 | Statistical analysis

Given the lack of data available in the literature at the time of planning this study, a power calculation for sample size estimation was not performed. Instead, the sample size was arbitrarily chosen (n = 60) as the minimum number required for the type of statistical method. Data were analyzed on an intention-to-treat basis. Statistical analysis was performed using commercially available software (SAS Version 9.4, SAS Institute, North Carolina). Significance was set at $P < .05$. Patient characteristics are reported as median (quartile 1 [Q1]-quartile 3 [Q3]) or number (%) and were not statistically compared between groups.[26,27] Where multiple RBC bags were administered to an individual dog, RBC bag age and IL-8 concentrations were averaged across all bags administered. The average then was used for data analysis. If a dog was missing 24 hours biomarker data because of death or discharge, RBC bag age and IL-8 concentration were excluded from analysis for any RBC bags transfused after 8 hours. Because of highly skewed data, RBC bag ages and IL-8 concentrations were compared between groups using the Wilcoxon rank sum test, and data are reported as median [Q1-Q3].

Distribution of body temperature and biomarker outcomes was assessed by visual inspection of histograms and Q-Q plots. Log transformation was performed for right skewed data to approximate a normal distribution. Data were described as mean (95% confidence interval [CI]), or geometric mean (95% CI) for skewed data. Concentrations of leukocytes, IL-6, IL-8, MCP-1, and CRP, and body

temperature were compared between groups over time using linear mixed effects models, with random effect of dog nested within treatment. A difference between groups in change over time was considered if a significant treatment-by-time interaction was identified. Clinical outcome data were not compared because of inadequate sample size for this analysis, but summarized data are provided.

3 | RESULTS

A total of 139 dogs were prescribed RBC transfusions and hence eligible for inclusion between July 15, 2017, and January 5, 2019, at MU and UQ. Seventy-two dogs were excluded, leaving a total of 67 dogs enrolled in the study (Figure 1). Sixty-one dogs (NLR, n = 27; LR, n = 34) had completed data collection through 8 hours and therefore were included in the final analysis.

3.1 | Patient and transfusion characteristics

Characteristics of the dogs in each group can be found in Table 1. The most frequent reasons for being prescribed a RBC transfusion were bleeding from an intra-abdominal mass and trauma (Supporting information, Appendix 2, Tables 1S and 2S).

Overall, 41 LR RBC bags and 37 NLR RBC bags were transfused (Table 2). Sixty-one of these blood bags were sourced from greyhound donors (31 LR, 30 NLR), of which 48 were from in-house or ex-racing greyhounds (25 LR, 23 NLR), and 13 were from community donors (6 LR, 7 NLR). The remaining 17 bags were from community donors of a variety of non-Greyhound breeds (10 LR, 7 NLR). No significant difference in age of transfused RBC bags was found between groups ($P = .22$). Interleukin-8 concentration was significantly higher in NLR RBC bags compared to LR RBC bags ($P < .001$, Table 2). All dogs received all RBC bags between 0 and 8 hours, with the exception of 4 dogs (1 LR, 3 NLR). The LR group dog received 1 bag between 8 and 24 hours, but was not alive for 24-hour sampling. Two NLR group dogs received 1 bag and 1 NLR group dog received 2 bags between 8 and 24 hours, and all were sampled at 24 hours. All dogs except 1 received RBC only from their allocated group. This protocol violation was a dog in the LR group that received a total of 4 RBC bags. Three LR bags were given per protocol, but 1 NLR RBC bag was transfused over 45 minutes as the fourth and final RBC bag. In this patient, the 8-hour sampling time point occurred incidentally immediately after the final transfusion, and because this dog was euthanized before 24 hours, no data were collected beyond 8 hours.

3.2 | Inflammation biomarkers and body temperature

Nine dogs in the LR group and 2 dogs in the NLR group did not have data collected at the 24-hour time point, because they had been euthanized (n = 8), or discharged (n = 3). No significant differences
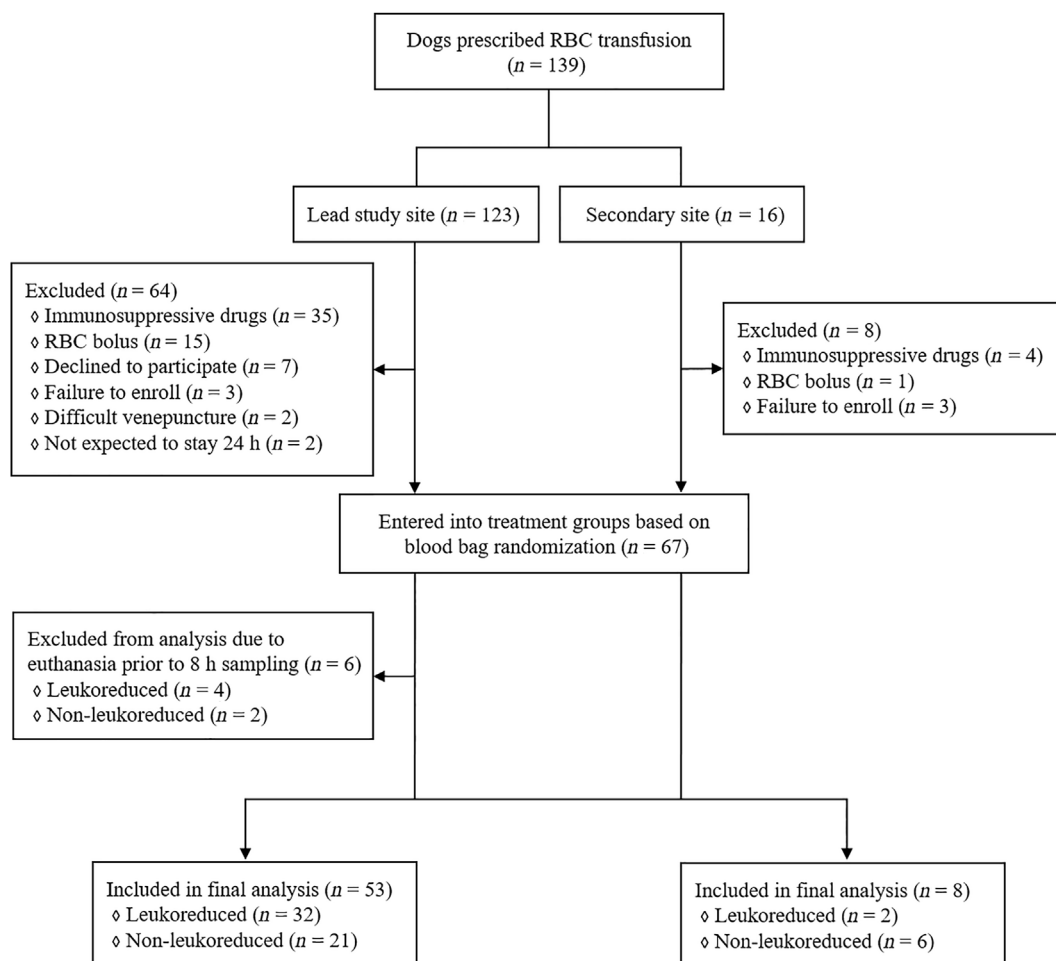
FIGURE 1 Flow chart of dogs included and excluded from randomization into a clinical trial at two participating hospitals to receive leukoreduced or nonleukoreduced red blood cells (RBC). h, hours

TABLE 1 Characteristics of dogs randomized to receive leukoreduced or nonleukoreduced red blood cell (RBC) transfusions

Characteristic	Leukoreduced (n = 34)	Nonleukoreduced (n = 27)
Age (years)	10.5 [6.9-11.6]	10 [5-11.3]
Sex		
Male intact	5 (14.7)	3 (11.1)
Male neutered	12 (35.3)	12 (44.4)
Female intact	4 (11.8)	6 (22.2)
Female spayed	13 (38.2)	6 (22.2)
Compatibility testing		
Blood typed	31 (91.2)	26 (96.3)
Cross-matched	10 (29.4)	2 (7.4)
Reason for transfusion		
Bleeding intra-abdominal mass	18 (53)	9 (33)
Trauma	7 (21)	12 (44)
Coagulopathy	5 (15)	3 (11)
Nonregenerative anemia	4 (12)	3 (11)

Note: Data are presented as either median [Q1-Q3] or number (percentage).

TABLE 2 Red blood cell (RBC) bag characteristics and transfusion logistics for dogs randomized to receive leukoreduced or nonleukoreduced RBC

|	Leukoreduced (n = 34)	Non-Leukoreduced (n = 27)
Age of transfused RBC bags (days)[a]	21 [13-31]	25 [21-34]
RBC bag interleukin-8 (pg/mL)[b]	19 [7-40]	266 [200-438]
Dogs transfused from only 1 RBC bag	30 (88)	23 (85)
Dogs transfused with >1 RBC bags	4 (12)	4 (15)
Dogs that finished all RBC transfusions within 8 hours[c]	33 (97)	24 (89)
Volume of RBC transfused (mL/kg)	13.6 [10.5-21.1]	13.3 [10.7-17.6]

Note: Data are presented as either median [Q1-Q3] or number (percentage).
[a]Compared between groups using Wilcoxon rank sum test, $P = .22$.
[b]Compared between groups using Wilcoxon rank sum test, $P < .001$.
[c]Blood sampling for inflammation biomarkers occurred before the first RBC transfusion started, then 8 and 24 hours after the first transfusion started.

FIGURE 2 Inflammation biomarker concentrations including A, leukocyte count; B, C-reactive protein; C, interleukin-6; D, interleukin-8; and E, monocyte-chemoattractant protein-1, over time in dogs receiving a transfusion of either leukoreduced (LR, n = 34) or nonleukoreduced (NLR, n = 27) red blood cells. Blood samples were collected within 1 hour of transfusion (0 hour), then 8 and 24 hours after the start of transfusion. Nine dogs in the LR group and 2 dogs in the NLR group were not sampled at 24 hours.
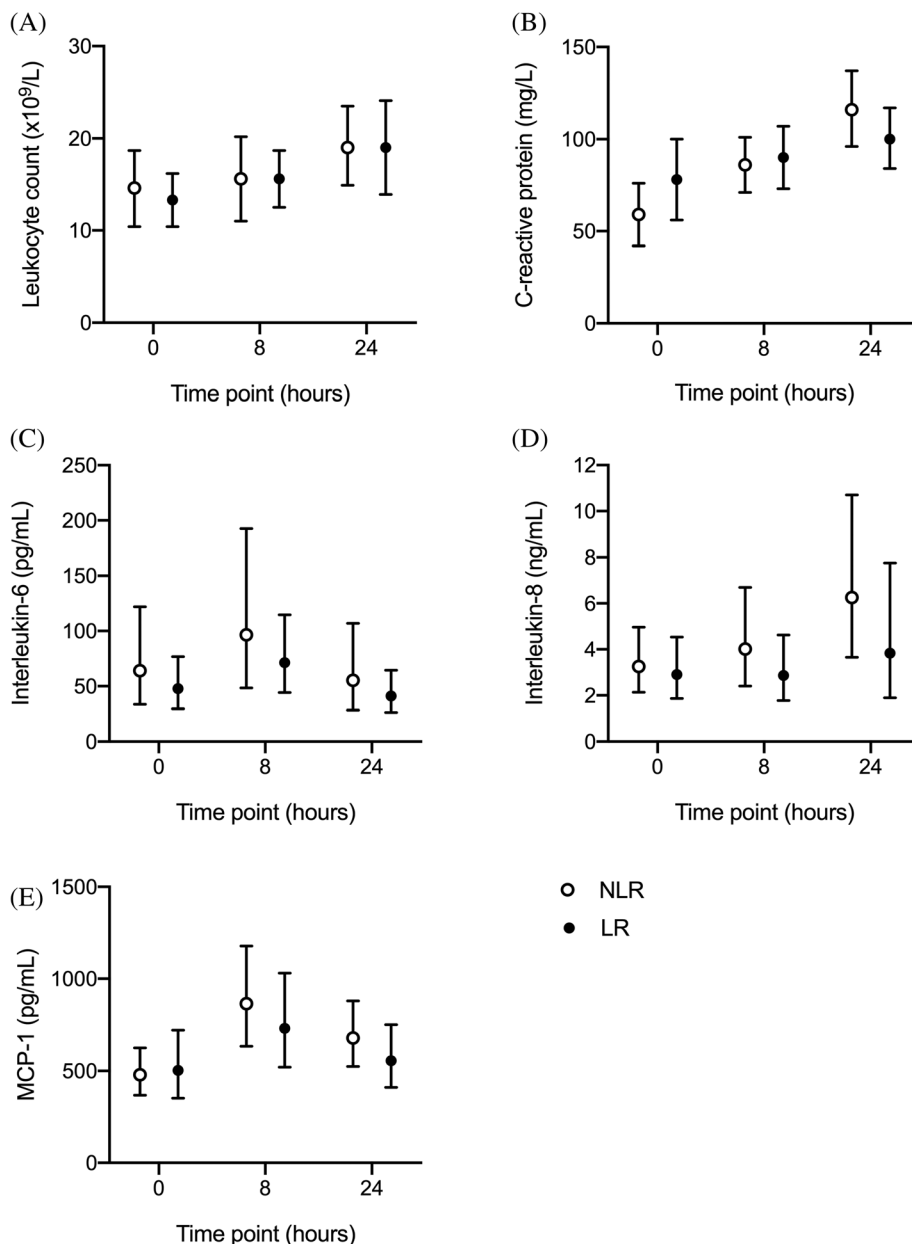
were found between treatment groups in change over time of leukocyte count (P = .93), CRP (P = .18), IL-6 (P = .99), IL-8 (P = .75), or MCP-1 (P = .69, Figure 2). No dogs had rectal temperatures ≥39.2°C (>102.5°F) at 0 hour. Two dogs in each group had rectal temperatures ≥39.2°C (>102.5°F) at either 8 or 24 hours, or at both time points. No significant difference was found between groups in change of temperature over time (P = .12, Figure 3). Ten dogs were removed from temperature analysis because of missing data (LR: n = 7; NLR: n = 3).

3.3 | Clinical outcomes

Ten dogs (10/34, 29%) in the LR group and 4 dogs (4/27, 15%) in the NLR group were euthanized, with 6 LR RBC recipients and 2 NLR RBC recipients euthanized before the 24-hour sampling time point.

No natural deaths occurred. Excluding euthanized dogs, the median duration of hospitalization for dogs in the LR and NLR groups were 2 (1–4) days and 3 (2–4) days, respectively.

4 | DISCUSSION

We developed a successful protocol to enroll and randomize critically ill dogs into a 2-center, blinded, placebo-controlled clinical trial to assess the effect of pre-storage leukoreduction on post-transfusion inflammation. We found no significant difference in change of inflammation biomarker concentrations over time between LR and NLR RBC recipients, possibly because of biomarker concentration variability, small sample size, and drop out of some dogs before 24 hours. However, importantly, we have reported the feasibility outcomes for a
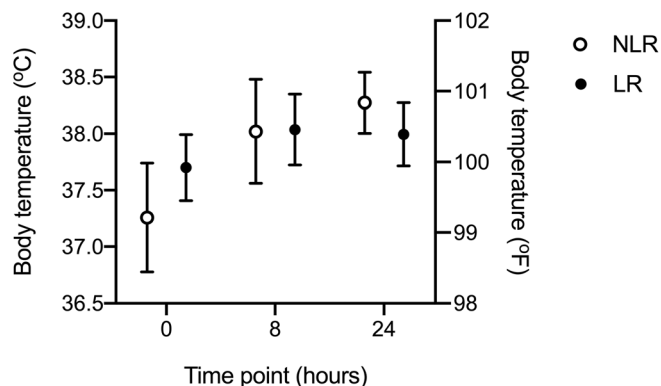
FIGURE 3 Rectal temperature over time in dogs receiving a transfusion of either leukoreduced (LR, n = 27) or nonleukoreduced (NLR, n = 24) red blood cells. Temperature was assessed immediately before the start of transfusion (0 hour), then 8 and 24 hours after the start of transfusion. Because of missing data, 10 dogs were excluded from analysis (LR, n = 7, NLR n = 3). There was no difference between groups over time ($P = .07$).

study protocol that can now be used to power and design a larger follow-up multicenter clinical trial.

Our trial protocol was novel in that the dedicated blood bank technicians randomized the allocation of RBC bags into LR or NLR at the time of blood collection, with no additional randomization occurring at the patient level. We found that this protocol allowed for easy patient enrollment with no breach in blinding. With our method, both LR and NLR bags, of both DEA 1 positive and negative blood, were readily available to use within the blood bank. Dogs prescribed a transfusion received the oldest compatible available RBC bag, which allocated each dog to 1 of the 2 study groups. Because all bags were clearly marked A or B, but otherwise identical in appearance, 1 dog could receive multiple bags of blood from within its original group allocation. This randomization and blinding protocol prevented the main enrollment problems seen in similar clinical trials, including missed enrollment because of lack of LR blood present in the blood bank,[21] and excluded dogs because of requirement for multiple transfusions.[21,22] Although our protocol led to all RBC-transfused dogs at the study sites receiving blood that had been prepared for the study, regardless of their enrollment in the study, the clinical balance between LR and NLR RBC precluded this feature from being of ethical concern. In consideration of planning a larger multicenter clinical trial focused on clinical outcomes, our protocol would be easy to implement at any veterinary institution with its own blood bank and dedicated blood bank technician.

Substantial heterogeneity was observed in concentrations of inflammation biomarkers in the transfusion recipients in our study. This finding, coupled with missing data at 24 hours because of euthanasia or discharge, likely decreased our ability to detect differences between groups over time. Although a previously published preclinical trial showed a clear increase in inflammation biomarkers from baseline after transfusion of NLR RBC that was attenuated with LR RBC, the dogs in that study were healthy and had minimal baseline variation in

inflammation biomarkers.[5] Illustrating the challenge introduced with heterogeneity, a similar small clinical trial in critically ill dogs with widely variable baseline biomarkers of inflammation also was unable to detect a difference in post-transfusion inflammation between dogs transfused LR or NLR RBC.[21] Unfortunately, these data were not yet published at the time our clinical trial was being designed and could not be used to help power our study. Although several limitations including small population, baseline heterogeneity, and missing data points likely contributed to inability to reject our null hypothesis, our biomarker concentration and clinical outcome data will be valuable in planning a follow-up clinical trial while anticipating population variability.

Given our small sample size, we attempted to increase the potential of finding a difference between groups by excluding dogs that were receiving or were likely to start receiving immunosuppressive drugs that might attenuate a post-transfusion inflammatory response. Immunosuppressants, notably the commonly used class of glucocorticoids, suppress the acute inflammatory response by inhibiting gene transcription of a variety of pro-inflammatory mediators such as cytokines, chemokines, arachidonic acid metabolites, and adhesion molecules.[28,29] Inhibition of the production of pro-inflammatory cytokines, or downstream signaling molecules such as CRP, could decrease the magnitude of any difference between groups.[30] We felt it prudent to limit variables causing additional heterogeneity of biomarker concentrations wherever possible. Consequently, our results may not reflect the clinical relevance of transfusing LR RBC in a population including dogs with immunosuppression. However, despite this exclusion, we still found no significant difference in inflammation biomarkers between groups over time.

Our randomization and allocation protocols precluded us from controlling for the age of RBC bags transfused to enrolled dogs. Older stored RBC, as opposed to freshly stored RBC, may induce inflammation in transfusion recipients,[31,32] and leukoreduction may or may not attenuate this response.[1,4,5] Thus, not controlling for RBC bag age may have introduced more variability into our population's inflammatory response. However, we believe our trial best reflects clinical practice where variably aged blood will be available for any given transfusion and therefore would provide the most relevant data regarding the effect of LR on post-transfusion inflammation. The median age of transfused RBC bags in our study was not different between groups, at 21 (14–31) days for LR and 25 (21–34) days for NLR. From previous in vitro analyses, we expected accumulation of IL-8 in NLR but not in LR RBC bags over time, with the highest concentration in older NLR bags.[13,15] Not surprisingly, given that our RBC bags were a median of 3 weeks old, we found significantly higher concentrations of IL-8 in NLR compared with LR RBC bags. Despite the difference in concentrations of transfused IL-8, we found no difference in inflammatory biomarker concentrations between recipients of NLR and LR RBC. Although this finding may reflect that IL-8 is not the stimulus for a post-transfusion inflammatory response, it is also possible that it reflects type 2 error, as discussed above.

To optimize our ability to detect a difference between treatment groups while also ensuring ethical treatment of critically ill dogs, we

selected specific inflammation biomarkers and limited sampling time points based on previous transfusion studies in dogs. Leukocytes and CRP were chosen because transfusion of LR RBC, compared with NLR RBC, to healthy dogs attenuated post-transfusion increases in these variables.[5] We also chose to assess concentrations of IL-6 as the stimulus for CRP production,[30] IL-8 given its propensity to accumulate in RBC during storage,[15] and MCP-1 given evidence of marked increases in critically ill dogs.[33] As detailed in methods, the post-transfusion time points of 8 and 24 hours were chosen to maximize the likelihood of finding a difference in the chosen inflammation biomarkers while minimizing the number of blood collections.[1,5] It is possible that between-group differences in inflammation biomarker concentrations may have been missed because of missing 24 hours data in dogs euthanized or discharged before collection, or because of a peak falling outside of our selected time points. Also, it is possible we may have excluded relevant inflammation biomarkers from our analysis, such as tumor necrosis factor-α, IL-1α and IL-1β. Overall, including a larger population of dogs to account for missing data, including more frequent sampling times, and extending the spectrum of inflammation biomarkers measured may increase the likelihood of identifying differences between treatment groups in future studies.

We found no significant difference in the change in body temperature over time between groups. Body temperature was recorded to assess for the development of FNHTR. However, we had not considered the potential for dogs to have external factors contributing to body temperature changes. Because FNHTR had not been specifically defined in veterinary medicine at the time we designed our study, we used the definition commonly used in people: an increase in body temperature of at least 1°C during or shortly after a blood transfusion.[18] Several of our enrolled dogs developed a 1°C change in body temperature over the 24-hour study period, which would have fit that definition of FNHTR. However, many of these dogs were mildly hypothermic at baseline, as indicated by the summarized data in Figure 3. Both external active warming devices and resolution of shock would have contributed to increasing body temperature into the reference range. Also, although blood sampling occurred as directed at all time points until hospital discharge or death, substandard compliance occurred with recording temperature at these time points, leading to several missing data points. Finally, our study was likely underpowered to detect a between-group difference in development of FNHTR, given its low reported incidence.[6,22] Although many large retrospective studies in people have identified a significant reduction in FNHTR incidence after implementation of leukoreduction, the effect size was small.[34,35] Future studies investigating the incidence of FNHTR with administration of LR compared with NLR RBC in dogs should plan to enroll a larger number of dogs and use the definition for FNHTR recently published in the Association of Veterinary Hematology and Transfusion Medicine Transfusion Reaction Small Animal Consensus Statement: a body temperature >39°C coupled with an increase of at least 1°C from pre-transfusion body temperature during or within 4 hours of the completion of a blood transfusion, provided external warming, infection, and other types of transfusion reactions have been ruled out.[6] Doing so will account for any nonpyrexic changes in body temperature and provide better guidance as to the timeframe in which to monitor dogs for development of fever.

Although our study was likely underpowered to find a difference in post-transfusion inflammation biomarkers with the use of LR RBC, we propose that sample size for a follow-up larger clinical trial can be estimated using the CRP data from our study. Using the mean CRP from our LR and NLR groups at the 24-hour time point, and the SD of 50 mg/L from the NLR group, a sample size of 150 dogs per study arm would give 80% power to detect a difference in mean CRP between 116.2 mg/L (NLR) and 100 mg/L (LR) at α = 0.05. C-reactive protein is a preferred biomarker to measure in a large multicenter clinical trial for several reasons. Firstly, canine CRP assays are routinely available in many commercial veterinary laboratories, and can be measured during the patient sample collection phase. Secondly, CRP reliably increases within 24 hours after transfusion in healthy dogs receiving NLR but not LR blood.[5] This situation is different from the biomarkers IL-6, IL-8, and MCP-1, where no differences were found between healthy dogs transfused with LR and NLR RBC in a separate experimental study.[1] Finally, in people, CRP correlates with increased risk of organ failure and death.[36] If this observation holds true in dogs, CRP may become an important outcome measure in critically ill dogs receiving transfusions. Thus, it is recommended that a follow-up larger multicenter clinical trial compare CRP concentrations over time between critically ill dogs receiving LR or NLR RBC. A secondary objective of any follow-up study also might be to compare the incidence of FNHTR within each group using the consensus statement definition described above.[6]

In conclusion, we developed a novel randomization protocol and executed a successful 2-center, randomized, blinded, controlled clinical trial to assess the effect of leukoreduction on post-transfusion inflammation in critically ill dogs. Although our study confirmed that concentrations of IL-8 were significantly lower in LR than NLR RBC bags, we found no difference in the post-transfusion inflammatory response in dogs receiving LR or NLR RBC, as measured by leukocyte count, IL-6, IL-8, MCP-1, and CRP concentrations. Our results will be useful in designing and powering a larger follow-up multicenter clinical trial to assess the effect of LR on post-transfusion inflammation in critically ill dogs.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

HUMAN ETHICS APPROVAL DECLARATION

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.