Effect of leukoreduction on concentrations of interleukin-8, interleukin-1β, and tumor necrosis factor-α in canine packed red blood cells during storage

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OBJECTIVE
To measure changes in interleukin-8 (IL-8), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) concentrations in stored canine packed RBCs (PRBCs) over time and assess the effect of leukoreduction on these cytokine concentrations.

ANIMALS
12 anesthetized healthy Greyhounds.

PROCEDURES
1 unit of whole blood from each dog was processed into PRBCs. Half of each PRBCs unit was passed through a leukoreduction filter to produce a leukoreduced unit, and the remaining blood was kept as a nonleukoreduced unit. All units had a CBC performed on day 0 (day of collection) and were stored at 2° to 6°C. Samples were collected from leukoreduced and nonleukoreduced units on days 0, 10, 20, 30, and 37 and centrifuged; the supernatant was stored at –80°C until analysis. Canine TNF-α and IL-8 concentrations were assessed with a multiplexed genomic and proteomic biomarker analyzer, and canine IL-1β concentration was measured by ELISA.

RESULTS
Leukocyte counts were decreased by ≥ 99.9% in all leukoreduced units. Median TNF-α and IL-1β concentrations were not significantly different between leukoreduced and nonleukoreduced units and did not change significantly during storage; median IL-8 concentration was significantly higher in nonleukoreduced versus leukoreduced units on all days, and was greater at all time points after ≥ 10 days of storage than on day 0. Median IL-8 concentration in leukoreduced units did not increase during storage.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that leukoreduction was effective for the removal of leukocytes from canine PRBCs and prevented significant increases in IL-8 concentration during storage. Further studies are needed to evaluate whether leukoreduction reduces cytokine-associated complications of transfusion. (Am J Vet Res 2015;76:969–974)
The aim of the study reported here was to measure IL-1β, IL-8, and TNF-α concentrations in canine PRBCs during storage and to assess the effect of prestorage leukoreduction on cytokine concentrations. We hypothesized that canine nonleukoreduced PRBCs would have significantly higher concentrations of IL-1β, TNF-α, and IL-8 after 37 days of storage, compared with canine leukoreduced PRBCs.

Materials and Methods

Dogs
Blood samples used in the study were obtained from 12 healthy retired racing Greyhounds (7 males and 5 females; age range, 17 months to 3 years) that had been donated to the university. The dogs were determined to be healthy on the basis of a physical examination performed by a staff veterinarian and results of a CBC. All dogs had a DEA 1.1-negative blood type. The study protocol was approved by the university’s Institutional Animal Ethics Committee (R2564/13).

Blood collection
Each Greyhound was sedated with methadone (0.3 mg/kg, IM). A 21-gauge IV catheter was placed in a cephalic vein, and anesthesia was induced with alfaxalone (2.3 to 3 mg/kg, IV) and maintained with isoflurane (concentration, 0.5% to 2.5%) in oxygen delivered via an endotracheal tube. Anesthetic depth, heart rate, respiratory rate, and blood pressure were monitored during anesthesia. Manual positive-pressure ventilation was performed during periods of apnea.

The right lateral cervical region was clipped of hair and aseptically prepared. The skin was incised with a scalpel. The carotid artery was isolated and aseptically prepared. The skin was incised, and a 14-gauge IV catheter was inserted into the artery and capped with a sterile injection port. A commercial 14-gauge IV catheter was inserted into the artery and capped with a sterile injection port. A commercial 14-gauge IV catheter was inserted into the artery and capped with a sterile injection port. A commercial 14-gauge IV catheter was inserted into the artery and capped with a sterile injection port. A commercial 14-gauge IV catheter was inserted into the artery and capped with a sterile injection port.

The blood collection system was inserted into the capped port, and 450 mL of blood was delivered into the sterile collection bag, which contained 63 mL of a citrate, phosphate, dextrose, and adenine anticoagulant solution. Three units (total, 1,350 mL) of whole blood was collected from each dog prior to euthanasia. The dogs were euthanized via IV injection of pentobarbital sodium while under anesthesia. Only the first unit of PRBCs collected from each dog was used in the present study; the second unit was donated to the study hospital’s blood bank for clinical use, and the third unit was collected for use in a later research project. The plasma removed was frozen and also donated to the blood bank for clinical use.

The blood units were weighed on a gram scale and were then centrifuged for 20 minutes at 4°C, and a plasma extractor was used to transfer the plasma into an integrally attached satellite bag. The seal of the integrally attached bag containing 100 mL of an RBC preservative, additive solution, was then broken and the preservative was added to the unit of PRBCs. To reduce interindividual variation among samples, each PRBC unit was divided into 2 equal parts; one of these was passed through a leukoreduction filter into a separate storage bag to produce a leukoreduced unit, and the other half was passed unfiltered into a separate storage bag to produce a nonleukoreduced unit. Each leukoreduced and nonleukoreduced unit was weighed with a gram scale; the original bag weight was then subtracted to calculate the volume of blood lost to the leukoreduction filter. All 24 PRBC units (12 leukoreduced and 12 nonleukoreduced) were stored at 2°C to 6°C and were mixed by inversion every 24 to 48 hours.

Sampling of prepared PRBC units
A blood bag sampling spike was aseptically inserted into each leukoreduced or nonleukoreduced PRBC bag (n = 24) port for repeated sample collection during the study. A 12-mL sample was aseptically collected from each bag immediately prior to storage (day 0); 2 mL was used for a CBC with an automated hematology analyzer; the remaining 10 mL was centrifuged for 15 minutes, and the supernatant was stored at –80°C. Additional 10-mL samples were collected aseptically from each bag on days 10, 20, 30, and 37. All of these samples were centrifuged as described, and supernatants were stored at –80°C for later cytokine analysis.

Blood collection was performed over a 2-month period. After the final sample was collected from each bag, the units were kept stored at 2°C to 6°C, and once all samples had been collected, an additional 4-mL sample was aseptically collected from each bag. Each final sample was transferred to a culture medium containing 20 mL of peptone-enriched tryptic soy broth supplemented with brain-heart infusion solids and activated charcoal and sent to a microbiology laboratory for aerobic and anaerobic bacterial culture.

Cytokine assessment
Concentrations of TNF-α and IL-8 were assessed with a canine cytokine magnetic bead panel in an in-house multiplexed genomic and proteomic biomarker analyzer. Canine IL-1β concentration was measured with an in-house ELISA. These tests were performed according to the manufacturers’ guidelines. A calibration and verification procedure was performed prior to sample analysis. Quality controls and serial dilution standards were prepared according to the manufacturers’ guidelines. The quality controls were within the appropriate range for each assay.

Statistical analysis
Statistical analysis was performed with commercially available software. Prior to data collection, a power calculation was performed to estimate adequate sample size for the study. Sample size estimations were performed with the level of significance set at α = 0.05 and power set at 0.80 (β = 0.20) to test the hypothesized effects between stored leukoreduced and nonleukoreduced PRBCs. It was expected...
that leukoreduced PRBCs would have negligible concentrations of these cytokines, and therefore a paired sample size of 4 was considered adequate to demonstrate that a response was significantly different from zero. To demonstrate a significant difference over time, expected effect sizes (difference in means/SD) of 0.25 to 1.0 were estimated from previous research in human medicine.5-12 Paired sample sizes of 128 to 10 would be required to detect these, respectively. A sample size of 12 was chosen to detect an effect size of at least 1.0, which was estimated as the expected difference between days 0 and 37.

Bag weight and Hct of each leukoreduced and nonleukoreduced unit were normally distributed, verified by failure to reject the null hypothesis of normality on a Shapiro-Wilk test (P > 0.05), and analyzed by means of a paired t test (P < 0.05). The concentrations of IL-1β, IL-8, and TNF-α were positively skewed and did not follow a normal distribution; the null hypothesis of normality was rejected by the Shapiro-Wilk test (P < 0.05). These data are presented as median and interquartile range (ie, 25th to 75th percentile). Square-root transformation of IL-1 and interquartile range (ie, 25th to 75th percentile). Square-root transformation of IL-1β and TNF-α concentrations created a normally distributed response on the basis of the Shapiro-Wilk test. The concentration of IL-1β, IL-8, and TNF-α was each analyzed with a mixed-effect model, evaluating the fixed effects of blood unit (leukoreduced or nonleukoreduced) and time (day 0, 10, 20, or 37) while accounting for the random variance of dogs across paired blood units and the repeated time points. Where there was significant interaction of blood units and time (at P ≤ 0.05), selected comparisons were made across blood units and time points against a Scheffé-adjusted P ≤ 0.05.

Results

The mean ± SD weight of each unit of PRBC (prior to aliquoting into 2 equal parts) was 400 ± 18 g. The mean ± SD weight of each leukoreduced unit was 180 ± 6 g, and that of each nonleukoreduced unit was 173 ± 13 g. There was no significant (P = 0.981) difference in mean weight between the leukoreduced and nonleukoreduced units. The mean ± SD weight of blood remaining in the leukoreduction filter was 47 ± 5 g. The Hct of leukoreduced units ranged from 0.61 to 0.71, and that of nonleukoreduced units ranged from 0.63 to 0.72. There was no significant (P = 0.336) difference in median Hct between the leukoreduced (0.68) and nonleukoreduced (0.70) units. Leukocyte counts in nonleukoreduced units ranged from 3.1 × 10⁶ cells/L to 10.7 × 10⁶ cells/L. All leukocyte counts in leukoreduced units were below the limit of detection for the hematology analyzer (< 20,000 leukocytes/L), indicating leukocyte reduction > 99.9% in all leukoreduced units. Median platelet count was 203 × 10⁴ platelets/L (range, 174 × 10⁴ platelets/L to 336 × 10⁴ platelets/L) in nonleukoreduced units and 3 × 10⁴ platelets/L (range, 2 × 10⁴ platelets/L to 8 × 10⁴ platelets/L) in leukoreduced units, indicating platelet reduction of > 96% in all leukoreduced units. Aerobic and anaerobic bacterial cultures for each of the leukoreduced and nonleukoreduced units yielded no growth.

Median IL-1β concentrations were not significantly (P = 0.351) different between leukoreduced and nonleukoreduced units and did not change significantly within unit type over time (Table 1). Median IL-8 concentrations were significantly (P < 0.001) lower in nonleukoreduced units on day 0, compared with those on days 10, 20, 30, and 37. Median IL-8 concentrations in nonleukoreduced units were significantly (P < 0.001) higher than those in leukoreduced units at all time points, and median IL-8 concentration in leukoreduced units did not change significantly at any time point during storage. The TNF-α concentrations were below the minimum detectable value (6.1 pg/mL) in all nonleukoreduced and leukoreduced units at all time points.

Discussion

Results of the present study showed that median IL-8 concentration was significantly increased in non-leukoreduced canine PRBCs at 10 to 37 days of storage compared with the day 0 value, whereas median IL-1β and TNF-α concentrations were not. Additionally,

Table 1—Median (interquartile range) cytokine concentrations (pg/mL) at predetermined time points in leukoreduced and nonleukoreduced PRBC units created from whole blood samples collected from 12 anesthetized healthy Greyhounds.

<table>
<thead>
<tr>
<th>Variable</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukoreduced</td>
<td>17 (0–25)</td>
<td>17 (2–30)</td>
<td>15 (4–30)</td>
<td>17 (3–37)</td>
<td>13 (5–35)</td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonleukoreduced</td>
<td>1,606 (1,149–2,314)</td>
<td>4,005 (2,334–5,693)</td>
<td>4,838 (2,778–6,060)</td>
<td>3,637 (3,003–7,167)</td>
<td>4,346 (3,369–5,280)</td>
</tr>
</tbody>
</table>

Day 0 was the day of blood collection and plasma extraction to create PRBCs (1 unit/dog). Each PRBC unit was divided into 2 equal parts; one of these was passed through a leukoreduction filter to produce a leukoreduced unit, and the other was transferred to a separate storage bag and kept as a nonleukoreduced unit. All units were stored at 2° to 6°C and were mixed by inversion every 24 to 48 hours; samples (12 mL on day 1 and 10 mL on all other days) were collected through a blood bag sampling spike at the times indicated. Ten millimeters of each sample was centrifuged, and the supernatant stored at −80°C until analysis.

Within a column for a given cytokine, median concentration is significantly (P < 0.001) higher for nonleukoreduced units than for leukoreduced units. Within a row, median cytokine concentration is significantly (Scheffé-adjusted P ≤ 0.05) different from that on day 0.
median IL-8 concentration was significantly lower in leukoreduced units than in nonleukoreduced units on all days evaluated. These findings are in accordance with those of a recent study\textsuperscript{15} that measured IL-1\(\beta\), IL-8, and TNF-\(\alpha\) in canine PRBCs.

Interleukin-8 is one of a family of chemotactic molecules, the chemokines.\textsuperscript{16} It is released from many cells, including macrophages, neutrophils, endothelial cells, and mast cells, following inflammatory stimuli.\textsuperscript{18,19} Interleukin-8 is the primary activator of neutrophils; it induces adherence to endothelial cells and promotes degranulation and respiratory burst.\textsuperscript{19,17,18,20}

It is unclear whether transfusion of blood products with high concentrations of IL-8 is associated with greater patient morbidity. In 1 study,\textsuperscript{21} effects of IL-8 administration to healthy primates were minor, including initial granulocytopenia followed by granulocytosis with no notable changes to hemodynamic parameters. Postmortem histologic evaluation in those primates showed increased neutrophil margination in the capillaries of the lungs, liver, and spleen, but there were no secondary lesions associated with the presence of these cells.\textsuperscript{21} Although no adverse effects were seen in healthy primates,\textsuperscript{21} it is possible that increasing activation, margination, and degranulation of neutrophils may potentiate organ damage in patients with systemic disease.

The present study revealed markedly higher concentrations of IL-8 in nonleukoreduced PRBCs than have been reported in stored human or canine blood products.\textsuperscript{9–11,15,22} On day 37, nonleukoreduced PRBC units had a median IL-8 concentration of 4,346 pg/mL. A study\textsuperscript{1} of human blood products found a mean IL-8 concentration of 745 \(\pm\) 710 pg/mL in PRBCs after 4 weeks of storage. The maximum IL-8 concentration measured in canine nonleukoreduced PRBCs in the study by Corsi et al\textsuperscript{15} was approximately 1,300 pg/mL after 35 days of storage. It is unclear why the present study contained more IL-8 concentration of 745 than twice the concentration of IL-8, compared with nonleukoreduced PRBCs, in the present study, IL-8 was synthesized rapidly and early, with significantly greater concentrations in nonleukoreduced units, from day 0 onward. It is theoretically possible that a genetic tendency in Greyhounds exists, causing early activation of leukocytes with greater synthesis of IL-8, compared with other dog breeds. Further research may determine whether breed-specific differences in cytokine production exist and whether different sedation and anesthetic protocols during blood collection affect cytokine production.

Median TNF-\(\alpha\) and IL-1\(\beta\) concentrations did not increase significantly at any time point during storage of leukoreduced or nonleukoreduced PRBC units, consistent with findings in the study by Corsi et al.\textsuperscript{15} Failure to find a significant difference in these concentrations may have been attributable to small sample size. In contrast to the previous study\textsuperscript{15} of canine blood products, results of the present study revealed a TNF-\(\alpha\) concentration below detectable limits in all PRBC units at all time points. Studies\textsuperscript{9–12,22} of human blood products have shown variable TNF-\(\alpha\) and IL-1\(\beta\) concentrations in PRBCs. A potent pyrogen, IL-1\(\beta\) is one of the major cytokines implicated in febrile nonhemolytic transfusion reactions in people.\textsuperscript{9–12} If the lack of significantly increased IL-1\(\beta\) concentrations at different storage times in the present study is representative of blood products from other canine populations, this may explain why a febrile response after transfusion has rarely been reported in dogs. Authors of a recent retrospective study\textsuperscript{27} of dogs that underwent PRBC transfusions reported an incidence of 51 of 211 (24\%) for febrile nonhemolytic transfusion reactions; however, the criterion to diagnose a febrile nonhemolytic transfusion reaction in that study was a rectal temperature of \(\geq 39^\circ C\) during or after PRBC transfusion. This is in contrast with the definition of febrile nonhemolytic transfusion reactions in people, which is defined as an increase in body temperature of \(\geq 1^\circ C\) associated with transfusion.\textsuperscript{28} Because an absolute temperature was used instead of a change in temperature, the study in dogs\textsuperscript{27} may have overreported the occurrence of febrile reactions, given that some of the dogs may have had a high body temperature prior to transfusion commencement. The frequency of febrile nonhemolytic transfusion reactions in people receiving leukoreduced PRBC transfusions is variable. In 2 studies, the frequency was as low as 0.4\% (60/16,246 and 78/22,387),\textsuperscript{29,30} and in a third study as high as 6.8\% (8/117).\textsuperscript{31} These reactions are a common cause of morbidity in people receiving transfusions, who experience considerable discomfort with the onset of fever and rigors.\textsuperscript{31} A number of issues make determination of the frequency of nonhemolytic transfu-
leukoreduction in blood products from dogs. Brownlee et al.33 effectively leukoreduced canine PRBCs and reported no adverse effect on the viability of RBCs that had undergone filtration. In our study, leukoreduction was successful at reducing leukocyte concentrations in all treated samples to below the limit of detection of the automated hematology system used (< 20,000 leukocytes/L). The US FDA recommends a residual leukocyte count of < 5.0 × 10⁶ leukocytes/unit of human PRBCs.34 Investigators in other studies have performed manual leukocyte counts to more accurately determine residual leukocyte counts. However, our leukocyte counts were well below FDA recommendations for human samples, and the automated counts were considered adequate to demonstrate successful leukoreduction.

Leukoreduction prevented significant increases in median IL-8 concentration in canine PRBCs in our study. Other studies35,36 have shown leukoreduction to reduce concentrations of other bioactive substances, including myeloperoxidase, eosinophil cationic protein, histamine, and plasminogen activator inhibitor-1, in human transfusion products. In blood products from dogs, leukoreduction was found to prevent the accumulation of inflammatory microparticles in one study37 and of vascular endothelial growth factor in another.38 Other benefits of treatment with leukoreduced, versus nonleukoreduced, blood products in human transfusion medicine include a lower incidence of febrile nonhemolytic transfusion reactions13,28–30,39 lower mortality rates,13,14 lower incidences of transfusion-related acute lung injury40 and postoperative infections,41 and lower rate of transmission of infectious disease agents such as cytomegalovirus.42 Leukoreduction increases the cost and preparation time of producing PRBCs. However, in light of preventing accumulation of IL-8 and the potential for removal of other inflammatory mediators not yet evaluated, leukoreduction may be cost effective if it reduces patient morbidity and mortality rates. Further large-scale clinical studies are needed to determine the role of inflammatory mediators on adverse effects of transfusion in dogs.

One strength of the present study was the low variability between leukoreduced and nonleukoreduced units where these were produced from the same unit of PRBC, but the sole use of Greyhounds for our blood units may limit the generalizability of our results. We do not know how concentrations of IL-8 or other cytokines in blood products from Greyhounds compare with those from dogs of other breeds. Despite this important limitation, the results obtained are worthy of consideration. Greyhounds are often used as blood donors because their blood antigen profiles more commonly make them universal donors, compared with dogs of other breeds.43 These dogs typically have a high PCV,44 and thus the products collected are RBC rich. The generalizability of our results may also be restricted because the dogs in our study were anesthetized and many received positive-pressure ventilation, which may have influenced cytokine release. Although our study was sufficiently powered to detect differences in IL-8 concentrations, it was underpowered to detect differences in IL-1β concentrations between nonleukoreduced and leukoreduced units or in nonleukoreduced units over time. The apparent differences in IL-1β were small and would require sample sizes > 40 to allow detection of significant differences. The clinical relevance of small differences, if any, is not known. Finally, we chose to assess differences in concentrations of specific cytokines in stored canine PRBCs on the basis of findings described for stored human PRBCs. There are likely other potential mediators of inflammation that could accumulate over time in stored canine blood products. However, owing to the clear difference in median IL-8 concentration between leukoreduced and nonleukoreduced units, the authors consider that leukoreduction would also likely reduce the accumulation of other, as yet uninvestigated, mediators of inflammation.

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The authors declare that there were no conflicts of interest.

Footnotes
a. Fenwal Whole Blood CPDA-1 Triple Blood-Pack Unit, Fenwal Inc, Lake Zurich, Ill.
b. Terumo Separation Stand, Teruflex, ACS-201, Terumo Corp, Tokyo, Japan.
c. Imugard III-RC Leukocyte Removal Filter, Terumo Corp, Tokyo, Japan.
d. Teruflex 150-mL transfer bag, Terumo Corp, Tokyo, Japan.
e. Take Set Swan-Lock needle-free multiple dose access device, Codan US Inc, Santa Ana, Calif.
g. BacT/ALERT PF Pediatric FAN, bioMerieux, Marcy l’Etoile, France.
h. Vetpath Laboratory Services, Ascot, WA, Australia.
i. Milliplex MAP Kit, EMD Millipore Corp, Billerica, Mass.
j. Magpix xMAP Luminex Corp, Austin, Tex.
l. SAS, version 9.4, SAS Institute Inc, Cary, NC.

References