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Immunologic profiles of red blood cells using *in vitro* models of transfusion

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Abstract

Background—Transfusion of packed red blood cells (RBCs) produces a myriad of immunologic derangements, from suppressive to stimulatory. Proliferation of human T cells is suppressed *in vitro* after exposure to processed red blood cells (PRBCs). We hypothesized that this effect would be mitigated by using fresh RBCs. We also hypothesized that this suppressive effect was a generalized effect on lymphocyte proliferation and would be observed in both CD4+ and CD8+ T-cell subpopulations as well as B cells.

Materials and methods—We isolated human T cells from donor peripheral blood mononuclear cells and exposed them to either blood bank PRBCs or fresh RBCs from volunteer donors and stimulated them with anti-CD3/anti-CD28. Human B cells were stimulated with lipopolysaccharide and exposed to PRBCs or fresh RBCs. We measured proliferation of B cells by thymidine incorporation assays. We also treated RBCs with citrate-phosphate-dextrose (CPD) at different time points before culture them with stimulated T cells to determine the role of this common RBC storage solution in lymphocyte proliferation.

Results—*In vitro* proliferation of CD4+ and CD8+ T cells was suppressed by blood bank RBCs. This suppression is eliminated when fresh RBCs were used. The B cells showed inhibition of proliferation when exposed to similar conditions, which appeared to be consistent over serial dilutions. Fresh RBCs exposed to CPD did not appear suppressive in the first 6 h after exposure.

Conclusions—T-cell and B-cell proliferation inhibition by blood banked RBCs suggests a generalized effect of RBCs on cellular proliferation. The lack of suppression by fresh RBCs further suggests that something involved in blood banking alters RBC properties such that they attain a suppressive phenotype. One such blood banking component, CPD, does not appear to affect this suppressive phenotype within the first 6 h.

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Keywords

Red blood cell; Transfusion; Immunomodulation; Critical care; Transfusion-related immunomodulation

Introduction

Although it is often necessary and life-saving in clinical practice, transfusion of packed red blood cells (PRBCs) alters the immune system, producing both proinflammatory and anti-inflammatory responses. Clinically, PRBC transfusion increases the risk of infection, length of stay, mechanical ventilation, and mortality [1]. One possible mechanism for these effects is that PRBC exposure suppresses T-cell proliferation, as was reported to occur in vitro in a dose-dependent manner without apoptosis or necrosis, in prior work from this laboratory [2]. Age of stored red blood cells (RBCs) has been shown to enhance both immunologic derangements and harmful postoperative outcomes in patients [3–5]. Studies have compared the effects of storage in RBCs <7 d old with RBCs stored for 7–30 d, but these studies used RBCs processed with conventional blood bank protocols [6–8]. Military use of warm fresh whole blood for transfusion has been associated with increased efficacy and decreased RBC storage lesions [9]. Murine studies indicate that fresh blood may abrogate some of the negative immune effects seen with stored RBCs, but fresh blood as a model has not been applied to human cells [6,10]. No studies of storage duration have yet isolated specific components and steps of preparation and storage, or measured their effects on immune parameters. Characterizing the changes induced in banked human RBCs, evaluating storage processes, and isolating components of the RBC storage process used in traditional banking may help identify a culprit component of suppressed T-cell proliferation and/or a chronologic point at which the “RBC storage lesion” of suppressed T-cell proliferation is triggered [6,11,12]. We previously showed that banked human RBCs suppress human T cells in a cell contact-dependent manner. However, human RBCs can suppress allogeneic or syngeneic human T cells (manuscript in preparation) as well as mouse T cells [2], which indicates a lack of specificity. The purposes of our study were to determine whether current blood bank processing contributed to the suppressive properties seen in stored RBCs, and to determine whether fresh RBCs could eliminate this effect. In the present study, we found that blood banked RBC could suppress the proliferation of both CD4+ and CD8+ T cells as well as B cells. In addition, we found that fresh RBCs lacked this suppressive property and that citrate-phosphate-dextrose (CPD) exposure of fresh RBCs had no effect within the first 6 h.

Materials and methods

This study was approved by the Institutional Review Board of the University of Kentucky Office of Research Integrity (Protocol 12-0966-P6 H).

T-cell isolation and culture

We performed T-cell isolation as previously described [2]. Human peripheral blood mononuclear cells (PBMCs) obtained from volunteer blood donors (Kentucky Blood Center,

Lexington, KY) using an institutional review board (IRB)–exempt protocol were separated using Ficoll-Hypaque gradient. We isolated T cells from PBMCs by depleting all non-T cells using a magnetic bead Pan T cell kit (Miltenyi Biotec, Auburn, CA) and an autoMACS separator, and plated them at 1.0×10^6 cells/mL in Roswell Park Memorial Institute 1640 culture media (Life Technologies, Carlsbad, CA) supplemented with penicillin-streptomycin, 10% heat-inactivated fetal bovine serum (FBS), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, henceforth termed CRPMI (complete RPMI). Then we stimulated the T cells with plate-bound anti-CD3 (1 μ g/mL, OKT-3; Ortho Pharmaceutical, Raritan, NJ) and anti-CD28 (100 ng/mL, Becton Dickinson, San Jose, CA). These studies require longer continuous incubation periods; thus, we further supplemented the media with minimal essential media nonessential amino acid solution, sodium pyruvate, and glutamine (CRPMI). We analyzed random samples of T cells by flow cytometry with CD4+ and CD8+ antibody stains to ensure the purity of the separated populations. All cultures were maintained at 37°C and 5% CO₂.

B-cell stimulation and culture

We cultured human PBMCs obtained from volunteer donors (Kentucky Blood Center) using IRB–exempt protocols at a concentration of 1.0×10^6 cells/mL in CRPMI media and stimulated B cells to proliferate by exposure to lipopolysaccharide (LPS) (Sigma, St. Louis, MO) at an initial concentration of 100 μ g/mL. Lipopolysaccharide stimulation results in isolated proliferation of human B cells, although the cell cultures themselves contained all components of human PBMCs. Cells were cultured in 96-well plates for 72 h at 37°C and 5% CO₂. We added ³H-thymidine for the final 18–20 h of incubation at 1 μ Ci/well, harvested cells on Perkin Elmer unifilter plates, and determined cell proliferation by scintillation counting.

Red blood cell cultures

To simulate transfusion, we obtained allogeneic human PRBCs (O[−]) from the University of Kentucky Hospital Blood Bank under an IRB-exempted protocol and added them to T-cell cultures. Following blood bank protocols, the PRBCs were leukocyte-reduced by filtration based on size and charge using a standard leukocyte-reduction filter that removes 99.9% of leukocytes. The PRBC units were not routinely irradiated. We added PRBCs (2- to 3-wk storage duration unless specified otherwise) to cultures. Initial hematocrit was 60%–80% in blood bank PRBC specimens used for experiments and was diluted in media to 2% by volume. The number of PRBC per well was standardized such that 2% hematocrit equated to 193 ± 17 PRBCs:1 T cell, and followed by serial dilutions. For carboxyfluorescein diacetate succinimidyl ester (CFSE) staining and culture, we counted RBCs by light microscopy using a hemocytometer and diluted them to an initial concentration of 2×10^8 /mL, followed by serial dilutions.

We obtained human T cells by following the protocol for T-cell isolation and culture, counted them, and suspended them in phosphate-buffered saline/0.1% fetal bovine serum at a concentration of 5.0×10^6 cells/mL. We prepared 5 mmol/L CFSE by dissolving one vial of stock CFSE solution (CellTrace, Grand Island, NY) with 18 μ L dimethyl sulfoxide. We then added CFSE at a volume of 0.25 μ L/ mL T cells, rapidly inverted them, and stained

them for 10 min at room temperature in the dark. Staining was quenched by addition of excess CRPMI. Cells were centrifuged at 300 g for 10 min at 18° C, supernatant was removed, and cells were recounted before suspension at a final concentration of 2.0×10^6 cells/mL. We stored CFSE-stained T cells in the dark at room temperature until they were ready for plating. To determine proliferation by CFSE dye dilution, harvested cells were stained with anti-CD4 Per CP and anti-CD8 PE (eBioscience, San Diego, CA) for 30 min at room temperature in the dark. Cells were then washed with and resuspended in phosphate-buffered saline/0.1% fetal bovine serum. Cells were placed on ice for immediate flow cytometry analysis.

Data analysis

Data collected after flow cytometric analysis included the percentage of total cells and percentage of proliferating cells. We did not compare numerical results across experiments, given the necessary use of different donor granulocyte units for each day. Each experiment contained unstimulated T-cell control conditions that allowed us to examine individual results essentially as a within-subject design. Results represent trends seen across multiple experiments. B-cell experiments rely on scintillation counts and are presented as the mean value of each condition, with standard deviations and standard error calculations performed in Microsoft Excel (Redmond, WA) databases.

Results

CD4 and CD8 T cells continue to proliferate after exposure to fresh RBCs

In thymidine incorporation assays measuring a variety of dilutions of PRBCs, human T cells stimulated with anti CD3/ CD28 did not proliferate after exposure to stored RBCs. Red blood cell concentrations of 100:1 (RBC:T cells), 50:1, 25:1, and 12.5:1, all showed near-complete suppression of T-cell proliferation [2]. In the present study, we used CFSE dye dilution as a direct measure of T-cell division in vitro. Purified T cells were CFSE labeled and stimulated with anti-CD3/CD28 in the presence of 100:1 blood banked or fresh RBCs. After 3 d, cells were harvested and stained for both CD4 and CD8, which allowed us to analyze both cell populations. In all conditions tested, PRBCs suppressed both CD4 and CD8 cell proliferation. When T cells were exposed to similar conditions using fresh RBCs that were leuko-reduced but unprocessed by blood bank standard protocols, CD4+ and CD8+ human T-cell proliferation continued uninhibited, similar to levels of positive control cells in various experiments (Fig. 1). Percentages of proliferating cells showed slight variation between experiments, but in multiple experiments using fresh RBCs, T cells showed proliferation similar to positive control conditions (T cells stimulated with CD3/CD28 and no RBC exposure). However, PRBCs suppressed T cells to proliferate at approximately one third of the positive control levels.

Blood bank storage processes

Published experiments implicate the blood bank storage process in the development of the RBC storage lesion; testing individual additives at varying time points will potentially elucidate the time at which RBCs become suppressive for T cells. To begin to assess the effect of additives, we exposed human T cells to fresh RBCs that had been treated with

CPD, an anti-coagulant solution added to donor RBCs during early processing by blood banks. Citrate-phosphate-dextrose is the traditional anticoagulant solution used to store donated blood in the blood bank. Red blood cells stored in additive solutions are known to have altered aging processes [13,14]. At time points ranging from 0 h (exposure to CPD and then immediate culturing) to 6 h after CPD exposure, RBCs were treated and then placed in culture with T cells. We saw no inhibition of proliferation after exposure to CPD-treated RBCs. This indicates that the change in the RBCs may happen later in the processing and/or that storage conditions other than the presence of CPD are responsible (Fig. 2).

Fresh RBCs alter B-cell proliferation

Because PRBCs inhibits CD4 and CD8 T cells, both syngeneic and allogeneic, as well as mouse T cells, we reasoned that proliferation inhibition might be a generalized effect on lymphocytes by PRBC. Thus, we examined human B-cell proliferation after exposure to both PRBCs and fresh RBCs. Using ^3H -thymidine incorporation to measure proliferation and LPS to stimulate proliferation of B cells from human PBMCs, we demonstrated suppression of proliferation after exposure to both PRBCs and fresh RBCs. We observed suppression of B-cell proliferation by 50% at RBC/PBMC ratios of 100:1, 50:1, 25:1, and 12:1 (Fig. 3). The overall magnitude of proliferation was low because of the weak stimulation of human B cells by LPS. These experiments are also limited because we did not use a pure human B-cell population. Thus, although these experiments suggest an inhibitory effect of PRBC on human B-cell proliferation, further studies better characterizing the effect of RBCs, both fresh and stored, on pure B-cell populations will be necessary.

Discussion

Transfusion-related immunomodulation persists despite leuko-reduction, which implicates the RBC itself as a major causative agent. Transfusion-related immunomodulation consists of both immunostimulatory and immunosuppressive phenomena. Proinflammatory responses (i.e., systemic inflammatory responses and multiple organ failure) are often seen after massive transfusions, whereas the immunosuppressive effects are seen in lower-volume transfusion of as small as a single unit of PRBCs. Likewise, the two-hit model of post-transfusion immune stimulation has been proposed, including an underlying inflammatory state that primes the recipient immune system with subsequent PRBC transfusion leading to marked proinflammatory responses [5,15]. This study clarifies known suppressive effects of PRBCs on human T cells, both CD4+ and CD8+, and demonstrates an ability to eliminate this phenomenon by using fresh RBCs not exposed to traditional blood banking methods. In examining the blood banking protocols, we were not able to induce a suppressive profile in RBCs after 6 h of exposure to CPD, which suggests that a CPD effect, if it occurs, does not develop immediately upon processing [16,17]. To further understand the suppressive effects of PRBC on different lymphocyte subsets, we have begun examining the effects of RBCs on human B cells. Early data indicate that both stored and fresh RBCs potentially suppress B-cell proliferation. Although these concepts require more detailed analysis, they offer some insight into the breadth of immunosuppressive mechanisms associated with transfusion-related immunomodulation. A previous study established that the immunosuppressive effects from transfusing a single unit of RBCs results in increases in surgical site infections,

length of stay, days of mechanical ventilation, and overall mortality [1]. Identifying the potential culprit in blood banking protocols offers great potential for modification of current practices in transfusion medicine. Further characterization of each component used in the process is under way.

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Fig. 1.

Packed red blood cells suppress proliferation of both CD4 and CD8 human T cells. Fresh RBCs restore proliferation of both CD4 and CD8 T cells. T cells were purified from human PBMCs, stained with CFSE, exposed to anti CD3/CD28, and then cultured with no RBCs, PRBCs (100:1), or fresh RBCs (100:1) for 4 d. Cells were then harvested, stained with anti-CD4 or anti-CD8, and analyzed by flow cytometry. Proliferation is assessed by the dilution of CFSE intensity.

Fig. 2.

Addition of CPD to RBCs does not suppress T-cell proliferation within the first 6 h after exposure. We added fresh RBCs to anti-CD3/CD28 stimulation cultures of CFSE-labeled T cells after the indicated times of incubation with CPD. T cells were harvested after 3 d and proliferation was measured by CFSE dye dilution using flow cytometry. Initial CFSE-stained T-cell populations are demonstrated by the large peaks at a fluorescence intensity of 10^2 ; the leftward diminution of fluorescence intensity demonstrates continued proliferation. Numbers in each histogram represent the percentage of T-cell division ($M2/M1 \times 100$).

Fig. 3.

Red blood cells have a suppressive effect on B-cell proliferation. B cells within human PBMCs were stimulated to proliferate by the addition of LPS at 100 $\mu\text{g/mL}$. Cells were then exposed to blood bank PRBCs and fresh donor RBCs at an initial concentration of 100 RBCs to one PBMC, followed by serial dilutions. Cells were cultured in 96-well plates with CRPMI media, pulsed with tritiated thymidine at 48 h, harvested, and read by scintillation counter at 72 h. Overall, cells exposed to RBCs showed significant inhibition of proliferation. No difference was appreciated between exposure to PRBCs *versus* fresh RBCs.