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T Cell Suppression by Red Blood Cells is Dependent on Intact Cells and is a Consequence of Blood Bank Processing

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Abstract

Background—Packed red blood cells (PRBC) suppress T cell responsiveness through a mechanism requiring cell-cell contact. Questions remain as to whether this effect is an allogeneic response, related to cell death, or dependent on particular components of the red blood cells.

Study Design and Methods—Peripheral T cells were isolated from healthy donors and exposed to stored allogeneic PRBC or autologous red blood cells after processing. Red blood cells were lysed by hypotonic solvent to produce cellular ghosts. Tritiated thymidine proliferation assays were utilized. Cultures were saturated with IL-2 to determine whether impaired IL-2 synthesis played a role.

Results—T cell proliferation was suppressed by both autologous and allogeneic PRBCs. PRBC membrane integrity does enhance T cell suppression. T-cell death is not responsible for the suppressive changes. IL-2 synthesis is suppressed in PRBC-exposed T cells but addition of exogenous IL-2 does not rescue proliferative capabilities. Proliferation of T cells was inhibited with PRBC exposure but mitigated with the addition of fresh RBC.

Conclusions—T cell suppression is enhanced by intact PRBC but this effect is unrelated solely to alloantigens. Neither apoptosis nor necrosis of T cells contributes to this phenomenon. IL-2 synthesis is suppressed after PRBC exposure as a consequence of T cell inhibition, but is not the primary cause of suppression. Fresh RBC do not mediate T cell suppression indicating that changes in the RBC and development of the storage lesion may occur during initial blood bank processing.

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Keywords

Transfusion; Immunomodulation; Red Blood Cell (RBC); Blood Bank

Introduction

Transfusion of packed red blood cells (PRBCs) has shown documented negative clinical effects, including infection, organ failure, and longer length of hospital stay.^{1–3} Current literature presents differing opinions regarding the true impact of RBC transfusion in randomized clinical trials.^{4–6} Diverse immunologic responses, varying from immunosuppressive to immunostimulatory, have been documented after PRBC transfusion and are characteristically grouped into the phenomena known as transfusion-related immunomodulation (TRIM).^{7–9} Over 15 million PRBC units are transfused annually in the United States, and some effects of TRIM can be documented after a single-unit transfusion.^{1,8,9} While the detailed mechanisms responsible for immune responses to transfusion remain unclear, increasingly restrictive clinical guidelines regarding transfusion of blood products are developing. Likewise, options such as autologous blood donation and near-universal leukoreduction of RBC units have become routine.¹¹ Despite these protective measures, evidence of TRIM persists in the transfused patient with a broad spectrum of severity and incidence depending upon host factors, volume transfused, storage duration, preparation method and clinical measurements.^{5,12,13} Several large multicenter trials are underway or recently completed, addressing issues of red blood cell storage age (RECESS),¹⁵ age of blood transfused to mechanically ventilated patients (ABLE),¹⁶ and age of blood cells in very low birth-weight infants (ARIPI).¹⁷ The ARIPI trial, published in October 2012, found no notable difference between newer stored blood cells (less than 7 days old) and standard, older stored blood used in neonatal intensive care units.¹⁸

We have previously reported a strong suppressive effect of PRBCs *in vitro* on human T cells resulting from direct cell-cell contact between the T cell and the PRBC.¹⁴ These studies raised the possibility that transfused RBC could have a negative impact on T cell function *in vivo*. In order to further understand this process, we studied the properties of the RBC required for T cell suppression as well as the causative effect on the T cell.

Materials and Methods

T cell Isolation and Culture

Human peripheral blood monocytes (PBMC) obtained from volunteer blood donors (Kentucky Blood Center, Lexington, Kentucky) using an IRB-exempt protocol were separated using Ficoll-Hypaque gradient. T cells were isolated from PBMC by depletion of all non-T cells using a magnetic bead Pan T cell kit (Miltenyi Biotec, Auburn, CA) and an autoMACS Separator and plated at 1.0×10^6 cells/ml. Routinely, random samples of isolated T cells were stained with anti-CD3, anti-CD4, and anti-CD8 antibodies and analyzed for purity by flow cytometry. T cell purity was always greater than 99%. Additionally, final cell numbers were ascertained by direct counting using light microscopy and hemacytometers. Cells were cultured in RPMI 1640 media (Roswell Park Memorial Institute, RPMI, Life

Technologies, Carlsbad, CA) supplemented with penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS), and further supplemented with MEM non-essential amino acid solution, sodium pyruvate and glutamine due to longer continuous incubation periods, hence forth termed CRPMI (complete RPMI). Cells were stimulated with plate bound anti-CD3 (1 μ g/ml OKT-3, Ortho Pharmaceutical, Raritan, NJ) and anti-CD28 (100ng/ml, Becton Dickinson, San Jose, CA). All cultures were maintained at 37°C and 5% CO₂. Stimulated T cells with no exposure to RBC or components were used as positive controls for each experiment.

Packed Red Blood Cell Cultures

To simulate transfusion units, allogeneic human packed red blood cells (PRBC, O negative) were obtained from the University of Kentucky Hospital blood bank under an IRB-exempted protocol. All units obtained, both autologous and allogeneic, were leukoreduced and stored in the standard solutions of citrate-phosphate-dextrose (CPD) and Optisol (crystalloid solution of sodium chloride, dextrose, adenine, and mannitol). None of the units were irradiated. PRBC (2–3 week (mean age 17 days) storage duration unless specified otherwise) were added to T cell cultures. Acceptable initial hematocrits (hct) for blood bank PRBC specimens used for experiments was 60–80% and samples were further diluted in CRPMI to 0.0365%–2% by volume as shown in figure legends. The number of PRBC per well were standardized such that 2% hematocrit equated to 193 $\pm$ 17 PRBC: 1 T cell and followed by serial dilutions.

Fresh Red Blood Cell Cultures

Fresh red blood cells were obtained from volunteer donors (of varying blood types) through an IRB- approved protocol (12-0966-P6H) by peripheral venipuncture using sterile technique. Approximately 2.5ml of venous blood was collected, centrifuged to remove plasma, separated over a Ficoll-Hypaque density gradient and washed of residual Ficoll to isolate erythrocytes. The RBC were then counted by light microscopy using a hemocytometer and diluted to initial concentrations of 2 $\times$ 10⁸/ml (100 RBC:1 T cell), followed by serial dilutions as in the PRBC experiments.

Proliferation Assay

Purified human T cells (1 $\times$ 10⁶/ml) with PRBC cultures were incubated in 96 or 24 well plates for a total of 72 hours. ³H-Thymidine was added for the final 18–20 hours of incubation at 1 μ Ci/well. Cells were harvested on Perkin Elmer unifilter plates and cell proliferation determined by scintillation counting.

Whole vs Lysed PRBC, Supernatant and PRBC Ghosts Preparations

Whole PRBC were treated exactly as in prior experiments. To obtain the lysed PRBC, allogeneic red blood cells were first diluted to appropriate hematocrits, frozen on dry ice and thawed two sequential times. Supernatants were obtained from PRBC segments by adjusting RBC hematocrit to the highest concentration to be tested, followed by centrifugation to pellet the RBC. Subsequent serial dilutions of supernatant were performed based on levels previously measured in the PRBC units (ranging from 0.55% to 0.01%). Intact PRBC,

PRBC lysates and supernatants were cultured with T cells for 48 hours prior to thymidine pulse.

To create PRBC ghosts, PRBC were washed in sterile PBS, initial hematocrit assessed by microcentrifuge and brought to 4% hematocrit. PRBC were suspended three times in 20ml of hypotonic 5mM sodium phosphate buffer, each time vortexed vigorously and centrifuged at 22,000G for 10 minutes. This ultracentrifugation permitted isolation of RBC membranes. Sealed ghosts were created by resuspending the pellet in 20ml PBS at 37°C for 40 min, then rinsing twice at 22,000g for 10 min with PBS. Sealed ghosts and PRBC membrane pellets were resuspended up to the original pretreatment volume of 0.5ml. Light microscopy was used to confirm the presence of ghosts. Ghosts were then placed in T cell cultures using conditions previously described.

Flow Cytometry

Apoptosis and viability were determined by Annexin V-FITC/PI staining of non-³H-thymidine treated T cells run in parallel with these proliferation assays. Briefly, following culture treatments, T cells were rapidly harvested, washed with binding buffer (10mM HEPES, 2.5mM CaCl₂, and 140mM NaCl, pH 7.4), adjusted to 1×10⁶ cells/ml and incubated with Annexin V-FITC for 15 minutes at room temperature. Following a brief wash with binding buffer, PI was added to cells.

IL-2 Treatment and Production

T cells were treated with serial dilutions of human IL-2 recombinant protein starting at 200 Units per well and analyzed for proliferation by tritiated thymidine incorporation. In addition, to assess IL-2 production, media was removed from T cells incubated for 48 and 72hrs in the presence or absence of PRBC and frozen at -80°C until analyzed. Human IL-2 in cell culture supernatants were analyzed by ELISA (OptEIA Set for human IL-2, BD Biosciences, San Diego, CA) per manufacturer's specifications. Briefly, 96 well plates were coated overnight at 4°C with capture antibody. The following day, the plates were washed and blocked for 1 hour with assay diluent at room temperature. The plates were then washed again and the standards or samples added for an additional 2 hours. After incubation the plates were gently aspirated, rewashed, and the working detector (detection antibody + SAV-HRP) added and allowed to incubate for 1 hour. This was followed by additional washes, a 30 minute dark incubation with substrate solution, finalized with the addition of the stop solution and absorbance read at 450nm on a spectrophotometer.

Statistical Analysis

Treatment groups and dilution groups within treatments were compared using analysis of variance (ANOVA) with post hoc Bonferroni comparisons. If heterogeneity of variance was confirmed with the Levene statistic, the natural logarithm of T-cell counts was used. If heterogeneity of variance persisted after log transformation, non-parametric Kruskal-Wallis tests were performed with non-parametric post-hoc group comparisons. A limited number of two-group sub analyses were performed using Student's t-tests. Confidence levels were set at p<0.05. Data were analyzed using SPSS version 20 software (IBM Corp., New York, NY).

Results

Allogeneic vs Autologous PRBC

Our previous studies showed that cell contact between PRBC and T cells is required for suppression of T cell proliferation. Thus, separating T cells and RBC with a semi-permeable membrane eliminated the suppressive effect, effectively ruling out any non-cellular blood bank component in the suppressive property. In addition, suppression occurred at very high dilutions of RBC, at RBC to T cell ratios as low as 10:1. Because the PRBC units were leukoreduced, the number of leukocytes present in the RBC + T cell cultures were negligible. Thus, we concluded that the T cell suppressive activity was due to RBC and not some other blood-derived component.¹⁴ Because the blood bank PRBC and T cells were obtained from different units and thus allogeneic, this raised the possibility that T cell suppression involves alloantigenic differences between RBC and T cell. To test this hypothesis, human T cells were placed in culture with autologous RBC obtained from the same granulocyte unit bag or allogeneic RBC from a segment of O(−) donor blood bags. Varying concentrations of donor RBC were added to autologous or allogeneic RBC, and proliferation assessed by tritiated thymidine incorporation. The autologous RBC showed inhibition profiles similar to both young (less than 24 hours since donation) and old (average 21 days since donation) allogeneic RBC (ANOVA $p = 0.843$) (Figure 1). In all conditions, proliferation recovers with 0.125% hematocrit RBC (Bonferroni P -values $> .05$ compared to positive control). This indicates that alloantigens are not required for T cell suppression after RBC exposure.

Intact RBC are required for T cell suppression

Although we demonstrated that cell contact is required for PRBC suppression of T cell proliferation, it was not clear whether intact PRBC were a requirement for this suppressive effect. In an attempt to isolate the contributing factors of RBCs to T cell immunosuppression, intact PRBC, lysed PRBC, supernatants from stored PRBC and RBC ghosts were compared. Replicating prior experiments, whole PRBC drastically reduced T cell proliferation, with significant effects seen as low as 0.25% hematocrit. Freeze-thaw lysis of PRBC immediately before adding to the T cell culture significantly reduced T cell proliferation but at 25–50% of the effect of whole PRBC in the 2% to 0.25% dilutions (Figure 2A). To determine whether any non-cellular component of the PRBC affected T cell proliferation, supernatant from stored PRBC was tested after pelleting the RBC. Consistent with our previous studies in which T cells and PRBC were separated by a semi-permeable membrane¹⁴, supernatants had no effect on T cell proliferation when obtained from 0.55 to 0.01% hematocrit. At 0.03%, supernatant from PRBC were somewhat stimulatory for T cell proliferation (Figure 2B). To further examine whether intact cellular membranes in the absence of cytoplasmic contents could mediate T cell suppression, T cells were exposed to intact PRBC, PRBC ghosts (lysed, re-sealed cells devoid of all cytoplasm), and PRBC membranes (lysed unsealed cells) and monitored for proliferation in response to anti-CD3/CD28. Inclusion of intact PRBC resulted in a dramatic suppression of T cell proliferation while sealed PRBC ghosts and PRBC membranes contributed only slightly to suppression of T cells, acknowledging the fact that RBC surface properties may change upon removal of cellular contents (Figure 2C). Thus, without cytoplasmic contents, PRBC membranes were

unable to effect suppression of T cell proliferation. Taken together, these results point to an important role of PRBC contents in mediating T cell suppression, suggesting that intact PRBC may be important for the delivery of PRBC contents in close proximity of the T cell.

T cell Suppression Is Not Associated with Cell Death nor Apoptosis

The suppressive effect of RBC on T cell proliferation could be due to either a block in activation/proliferation or to cell death. To distinguish between these two possibilities T cells were stained with Annexin V and propidium iodide after 4 days in culture exposed to PRBCs. Apoptotic cells were considered to stain positive for Annexin V, a marker identifying early-stage apoptotic cells with exposed phosphatidyl serine residues within intact membranes. Late stage apoptotic and necrotic cells stain positive for propidium iodide, which can permeate through the membranes of dead and irreversibly damaged cells. T cell cultures activated in the absence of PRBC underwent active proliferation over the 4 day period. As is typical for such cultures, there was a minority of the population undergoing apoptosis and necrosis (Figure 3). The viable cells in these cultures were large blasted lymphocytes as assessed by flow cytometry (not shown). In contrast, in the presence of PRBC, almost 100% of the cells were viable and the size distribution was indicative of small resting lymphocytes. This indicates that the inhibition of T cell proliferation by PRBC was not due to induction of apoptosis or necrosis.

IL-2 does not rescue T cell proliferation

IL-2 is an autocrine growth factor required for T cell proliferation *in vitro*. To assess if IL-2 production by stimulated T cells was altered after PRBC exposure, levels were measured from supernatant harvested from T cell/PRBC cultures at 48 and 72 hours. IL-2 was significantly depressed at both 48 and 72 hours in cultures containing PRBCs in comparison to normally stimulated T cells, and slightly elevated in comparison to unstimulated T cells (Table 1). Because IL-2 production precedes proliferation in activated T cells, these results indicate that PRBC suppression is mediated early in T cell activation. To determine if the lack of T cell proliferation was due solely to the inability to produce IL-2, we attempted to rescue proliferation by the addition of recombinant IL-2 to the cultures of T cells and PRBC. Addition of IL-2 to PRBC/T cell cultures did not significantly rescue T cell proliferation in the presence of RBC even though it enhanced proliferation of T cells activated in the absence of PRBC (Figure 4). Thus, although IL-2 production by T cells is inhibited by co-culture with PRBC, the lack of IL-2 alone is not responsible for the proliferation inhibition.

Suppression of T cell proliferation is a function of blood bank storage

After examining multiple components of the banked red blood cells and their effects on T cell proliferation, a comparison with fresh, unprocessed red blood cells was performed. Changes after blood-bank storage and processing have been well- reported.^{4,5,6,7,17,19,20} To test these changes as a culprit for T cell suppression, fresh RBC were directly compared to stored PRBC in T cell proliferation assays. When exposed to PRBC from four different donors at a concentration of 100 PRBC: T cell, T cells showed marked suppression. Use of fresh RBC from two different donors, however, resulted in proliferation levels nearing maximal stimulation (Figure 5). In comparing the proliferation of T cells exposed to PRBC

to the proliferation of T cells exposed to fresh RBC, all dilutions (from 100:1 to 12.5:1) demonstrate statistically significant differences ($p < 0.05$ for all conditions). These results indicate that a suppressive phenotype is acquired during blood bank processing of red blood cells.

Discussion/Conclusions

Immunologic derangements resulting from transfusion of banked blood are varied, including both pro- and anti-inflammatory effects. While we have previously established that exposure to packed red blood cells from traditional blood banks suppresses human T cell proliferative capabilities *in vitro*, we now show that these properties are dependent on intact red blood cells and appear to result from either processing or storage procedures. Prior experiments have demonstrated that the suppressive effect of PRBC on T cell proliferation requires direct cell-cell contact, as separation of the two cell types by 0.2µm membranes in culture resulted in restoration of proliferative capabilities.¹⁴

The possibility of alloantigenic differences between PRBC and T cells as a potential mechanism for transfusion-related immunomodulation has been proposed. While processed red blood cells used in transfusions are frequently subject to leukoreduction, a negligible amount of donor white blood cells (WBC) will remain with the donor unit. Prospective clinical studies examining the immunologic effects of leukocyte reduction remain unclear.¹⁹ Additional *in vitro* experiments have demonstrated that some immunologic effects remain even in supernatants from leukoreduced, stored PRBC units.²⁰ Exposure to the minimal remaining donor WBC and their accompanying alloantigens have long been considered as an inciting event for TRIM. We demonstrate that leukoreduced autologous PRBC processed and stored via current banking protocols suppress T cell proliferation in a manner similar to leukoreduced allogeneic RBC, eliminating alloantigens as a sole cause, even those present on PRBC, as a potential cause of the suppressive phenomenon. Prior studies using unprocessed RBC have also indicated that these effects occur without involvement of other cell types such as monocytes or B cells.¹⁹ These data may provide one reason that autotransfusion has not abrogated transfusion-related immunomodulation (TRIM) in clinical studies.

Elucidating precise components of PRBC responsible for T cell suppression prompted analysis of PRBC contents, membranes, ghosts, and intact cells. Red blood cells that were lysed and placed into culture without separating contents from membranes showed significantly less suppressive properties than whole PRBC, but were nonetheless suppressive. When supernatants were prepared that were devoid of RBC membranes, T cell suppression was eliminated. In other studies supernatant from stored PRBC units have been shown to have varying immunomodulatory effects depending on storage age of the unit. However, experiments on T cells with supernatants alone have failed to demonstrate any change in T cell activation.²⁰ Finally, when PRBC ghosts and membranes depleted of intracellular content were used, minimal T cell suppression was observed. To further rule out a role for other additives present in PRBC units, we have shown that individual components of blood bank processing (CPD, Optisol) alone do not suppress T cell proliferation in these conditions.

As seen in Figure 3, the absence of T cell proliferation in our studies is not attributable to cell death. The exposure to transfused red blood cells does not kill the proliferating T cell, but arrests proliferation while maintaining viability. Prior work by Arosa, et al also demonstrated that non-banked RBC were capable of protecting antigen activated T cells from undergoing apoptosis or necrosis.²¹ While the T cells remain viable, they show inability to produce anticipated levels of cytokines such as IL-2. Their proliferative capacity is not rescued by exogenous administration of IL-2, which serves to further implicate the red blood cell itself as the cause of suppression and the possible downregulator of IL-2 receptors expressed by activated T cells.

Previous reports have demonstrated conflicting results, showing that PRBC could actually induce proliferation of human T cells.²¹ These studies, however, use phytohemagglutinin for their T cell mitogen, which notably leads to RBC agglutination and could drastically alter their function.²¹ Our study uses anti-CD3/anti-CD28 to stimulate the T-cell receptor complex to represent a more physiologic means of stimulation and, we believe, much more accurately represents the physiologic activation of T cells *in vivo*.

Most remarkable, however, is the important finding that fresh RBC, unprocessed with blood banking procedures, do not demonstrate suppressive profiles. This suggests that the vital change in the PRBC occurs at a specific step in the traditional blood storage protocol. Blood banks often use additives including citrate-phosphate-dextrose (CPD) and Optisol (crystalloid solution containing sodium, dextrose, adenine, and mannitol), either of which could alter the red blood cell during the initial processing or as a cumulative or time-dependent effect during storage. This offers a potential explanation for the effects seen with blood units of varying ages.

Many of the previous studies of PRBC examine effects of length of storage, and often refer to “fresh” cells as those stored for only a few days (ranging from 1–14 days).²² These cells, however, have gone through the storage and processing procedures of the blood bank, and are thus subject to any changes induced along the way. Our study is unique in the fact that we use “fresh” RBC that have not been prepared for storage (other than drawing into standard collection tubes with minimal volumes of EDTA). Comparing these “fresh” RBC to stored PRBC *in vitro*, we have shown a marked distinction in the immunologic effects brought on by routine blood bank processing. We recognize that isolating the particular time or condition during processing which converts the human erythrocyte into an immunosuppressive tool is paramount. Likewise, determining if this effect is in any way reversible will have dramatic implications for the field of transfusion and the many patients dependent on donated blood. Of note, our study is limited in that all banked blood units are obtained from one local blood bank, and our fresh donor samples represent a small sampling of the population, potentially biasing portions of the results. Tremendous variation in donors is likely present, from the maximal proliferative ability of the T cells, to the blood type of the donors. We do, however, see similar results across numerous experiments, with the fresh red blood cells never appearing to suppress proliferation of the T cells. Likewise, further work from our laboratory has begun testing individual blood bank additives in an attempt to isolate components potentially responsible for the observed suppression.²³

Another limitation of these findings is that all experiments thus far are representative of *in vitro* phenomena. Clearly, this profound level of T cell suppression does not occur in humans following the typical transfusion. Our results highlight the possibility that transfused PRBC could exert more subtle, but significant effects on host T cells. Thus, further characterization of the effects of blood transfusion on T cells in an *in vivo* setting, both animal models and in humans is warranted. Rapid clearance of transfused red blood cells (often within the first hour after transfusion) has supported the hypothesis that changes occur during PRBC storage and may be proportional to storage time.²⁴ Analysis of PRBC clearance and potentially accelerated erythrocyte aging using both stored and fresh blood provides an interesting avenue for *in vivo* studies of storage-induced changes.²⁵ By isolating the time period in which a suppressive profile develops in the PRBC, we hope to identify a potentially reversible cause and attempt to eliminate this particular avenue of immunosuppression associated with blood transfusions.

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Figure 1. RBC alloantigens are not responsible for inhibition of T cell proliferation

Purified T cells were stimulated with anti-CD3/CD28 in the presence of media only (Pos Control) or in the presence of serially diluted PRBC, treated with tritiated thymidine at 48 hours, and harvested 18 hours later. Patterns of proliferation in stimulated human T cells are similar after exposure to autologous or allogeneic blood bank RBCs (ANOVA $p=0.843$). Recovery of proliferation occurs at a concentration of 0.125% PRBCs (Bonferroni P 's $>.05$ relative to positive control in all three treatments). In comparing autologous RBC to both fresh and older allogeneic RBC, T cell inhibition is not a function of alloantigens.

Figure 2A

Figure 2B

Figure 2C

Figure 2.

Figure 2A. Intact red blood cells are required for suppression of T cell proliferation. A) Purified T cells were cultured in media only (Neg control) or anti-CD3/CD28 in the absence (Pos control) or presence of different concentrations of whole or lysed PBRC.

Figure 2B. Purified T cells were cultured in media only (Neg control) or anti-CD3/CD28 in the absence (Pos control) or presence of different concentrations of PRBC supernatant.

Figure 2C. Purified T cells were stimulated with anti-CD3/CD28 in the absence (Pos control) or presence of intact RBC, RBC ghosts, or RBC membranes. Statistical significance was seen between positive control and intact reds, sealed ghosts ($p < 0.001$) and PRBC membranes ($p < 0.001$ for all conditions). Likewise, statistical significance was noted for intact PRBC when compared to sealed ghosts and membranes (p values < 0.001). No statistical significance was seen between sealed ghosts and membranes ($p = 0.494$).

Figure 3. Lack of T cell proliferation does not result from apoptosis or cell necrosis

T cells were stimulated with anti-CD3/CD28 for four days in the absence (dark bars) or presence (light bars) of PRBC, treated with Annexin V and Propidium Iodide and analyzed by flow cytometry to assess percentages of apoptotic or necrotic cells in culture. Apoptotic cells were defined as Annexin V (+) and PI (-). Necrotic cells were considered Annexin V (+) and PI(+). Cultures treated with blood bank PRBC showed minimal apoptosis or necrosis when compared to stimulated T cells alone ($p=0.005$, 0.015 for apoptosis and necrosis, respectively).

Figure 4. Addition of IL-2 does not recover proliferative capabilities of T cells exposed to PRBC
 T cells were cultured alone (Neg control) or with anti-CD3/CD28 without (dark bars) or with (light bars) 2% PRBC. Various concentrations of exogenous IL-2 or media alone (Positive control) were added. Addition of IL-2 to PRBC/T cell cultures did not salvage T cell proliferation to levels observed in positive control T cells ($p < 0.001$ for all conditions tested).

Figure 5. Fresh RBC lack the suppressive properties of PRBC

Purified T cells were cultured in 96-well plates with media alone (Neg Control) or anti-CD3/CD28 with no addition (Pos Control) or PRBC from four different blood bank units or fresh RBC from two different volunteer donors for 48 hours, pulsed with tritiated thymidine, harvested and read by scintillation counter 18 hours later. For each dilution, comparing proliferation of T cells exposed to PRBC with T cells exposed to fresh RBC resulted in statistically significant differences ($p < 0.05$ for all conditions). These experiments were repeated at least three times, using multiple fresh donors for each experiment.

Table 1
IL-2 production is inhibited by exposure to PRBC

IL-2 production was measured by ELISA at 48hr and 72hr after culture of T cells with blood bank RBCs. At 48 hours, stimulated T cells vs. stimulated T cells with blood bank PRBC showed a difference in IL-2 production ($p=0.008$), however this was no longer statistically significant at 72 hours ($p=0.163$). Significant differences were also noted between unstimulated T cells vs. stimulated T cells at 48 hours ($p=0.007$), and between unstimulated T cells and stimulated T cells + PRBC at 48 and 72 hours respectively ($p=0.003$; $p=0.003$).

Incubation Time	Unstimulated T cells	Stimulated T cells	Stimulated T cells + 2% PRBC
48 hr	<7.0	1004 $\pm$ 85	36 $\pm$ 2
72 hr	<7.0	91 $\pm$ 27	31 $\pm$ 1