Reticulocytes in donor blood units enhance red blood cell alloimmunization

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Abstract

Although red blood cell (RBC) transfusions save lives, some patients develop clinically-significant alloantibodies against donor blood group antigens, which then have adverse effects in multiple clinical settings. Few effective measures exist to prevent RBC alloimmunization and/or eliminate alloantibodies in sensitized patients. Donor-related factors may influence alloimmunization; thus, there is an unmet clinical need to identify which RBC units are immunogenic. Repeat volunteer blood donors and donors on iron supplements have elevated reticulocyte counts compared to healthy non-donors. Early reticulocytes retain mitochondria and other components, which may act as danger signals in immune responses. Herein, we tested whether reticulocytes in donor RBC units could enhance RBC alloimmunization. Using a murine model, we demonstrate that transfusing donor RBC units with increased reticulocyte frequencies dose-dependently increased RBC alloimmunization rates and alloantibody levels. Transfusing reticulocyte-rich RBC units was associated with increased RBC clearance from the circulation and a robust proinflammatory cytokine response. As compared to previously reported post-transfusion RBC consumption patterns, erythrophagocytosis from reticulocyte-rich units was increasingly performed by splenic B cells. These data suggest that reticulocytes in a donated RBC unit impact the quality of blood transfused, are targeted to a distinct compartment, and may be an underappreciated risk factor for RBC alloimmunization.

Introduction

Red blood cells (RBC) are transfused to correct anemia from bone marrow failure, hemolytic anemia, and hemorrhage. Although RBC transfusions can be lifesaving, some patients develop alloantibodies against donor RBC blood group antigens. These alloantibodies can be clinically significant, inducing hemolytic transfusion reactions and hyperhemolysis, raising transplantation barriers, and delaying transfusion by making it difficult to find compatible blood for transfusion.[1] Once alloantibodies arise, patient care becomes more challenging. Currently, there are few strategies to prevent RBC alloimmunization besides antigen matching or antigen avoidance, and few treatments are available (with limited efficacy) once alloimmunization occurs.[1]

As opposed to pharmaceuticals, each unit of RBC is unique as it comes from a distinct individual, undergoes multiple manufacturing steps, and then is followed by a variable duration of refrigerator storage up to 42 days before transfusion.[2,3] Increasing evidence suggests that the risk of alloimmunization following an RBC transfusion is influenced by donor factors (e.g., blood group antigen density), component factors (e.g., storage duration), and host factors (e.g., blood group antigen negativity, HLA type, inflammation).[4] Identifying new risk factors that can prevent and/or reduce risk of future RBC alloimmunization events is a priority, given the current lack of available treatments for sensitized patients. In both humans and mice, RBC alloimmunization rates increase in transfusion recipients with viral infections or viral-like inflammation.[4,5] Viruses induce robust type I interferon (IFN-α/β) production; in mouse models of RBC alloimmunization, blocking IFN receptors prevents alloantibody production, whereas

infusing IFN-α promotes alloantibodies.[6,7] Mechanistically, viral-like inflammation stimulates splenic antigen-presenting cells (APC) and alters the consumption of transfused RBC towards immunogenic dendritic cell (DC) subsets and away from red pulp macrophages.[8,9]

Intriguingly, patients at highest risk for RBC alloimmunization (e.g., those with sickle cell disease [SCD]) have elevated type I IFN levels.[10-12] Although the mechanisms underlying type I IFN production are unknown, we and others recently published that mitochondria in human RBC stimulate type I IFN production by immune cells.[13,14] Although mitochondrial retention in mature RBC is abnormal, most early reticulocytes have identifiable mitochondria. Some transfusion recipients and repeat volunteer blood donors have elevated reticulocyte frequencies.[15,16] Moreover, because compensatory reticulocytosis lasts longer than the interval between donations, RBC units from some repeat volunteer blood donors have reticulocyte counts above the reference range.[15] Of note, stress-induced reticulocytes produced in response to anemia (e.g., phlebotomy, hemorrhage) are larger, less deformable, and contain higher numbers of mitochondria.[17-23] Thus, we hypothesized that reticulocytes in RBC donor units may enhance RBC alloimmunization.

Using a murine model of RBC alloimmunization, we test the hypothesis that elevated reticulocyte counts in RBC donor units enhance RBC alloantibody production. We demonstrate a direct correlation between reticulocyte counts in an RBC donor unit and RBC alloantibody levels produced in transfusion recipients. Compared to controls, reticulocyte-rich RBC donor units were enriched in mitochondrial proteins and metabolites and, upon transfusion, elicited proinflammatory cytokines. Mechanistically, RBC from reticulocyte-rich units had lower 24-hour post-transfusion recovery (i.e., were cleared more robustly in the first 24 hours after transfusion) and induced significantly higher RBC consumption by splenic APC. Multiple B-cell subsets consumed most of the reticulocytes, representing a departure from normal post-transfusion RBC consumption patterns. The effect of higher reticulocyte frequencies was dramatic because transfusion of short- (i.e., 1 day) and medium- (i.e., 6 days) stored RBC donor units similarly induced alloantibodies. Lastly, we show that reticulocyte-mediated enhanced alloimmunization is not Toll-like receptor (TLR)-4 dependent. Together, these data demonstrate that reticulocytes in RBC donor units can enhance alloimmune responses.

Methods

Mice

HOD mice,[24] expressing an RBC-specific triple fusion protein consisting of hen egg lysozyme, ovalbumin, and human blood group molecule Duffy, were bred in the Columbia University vivarium. C57BL/6 (B6, strain #027) mice were purchased from Charles River Laboratories. C57BL/6-Tg(UBC-GFP)30Scha/J (GFP; strain #004353) and B6(Cg)-Tlr4$^{tm1.2Karp}$/J (TLR4$^{-/-}$, strain #029015) mice were purchased from The Jackson Laboratory. All mice were matintained on a 12:12 light/dark cycle and maintained on a chow diet (*ad libitum*) unless otherwise specified. All murine experiments were approved by Columbia University's Institutional Animal Care and Use (IACUC) committee.

Reticulocyte induction and blood bank preparation

Reticulocytosis was induced with phenylhydrazine (PHZ), or by iron deficiency followed by acute iron repletion. PHZ: intravascular hemolysis was induced with two intraperitoneal (i.p.) injections of PHZ (Sigma) 50 mg/kg/day separated by 24 hours or saline control. Iron: weanling (3-week-old) mice were placed on iron-deficient (Harlan Teklad TD.110592) or replete (Harlan Teklad TD.110593) diets for 4 weeks, followed by reticulocytosis induction by i.p. injection of 5 mg of iron dextran (Allergan NDC 00230608-10) and switching to iron-replete chow.[25]

Whole blood was collected by cardiac puncture into 14% CPDA-1 4 days after PHZ or iron dextran injection, pooled, filter leuko-reduced (Acrodisc WBC syringe filter, Pall), packed to 60% hematocrit, and stored for 1 or 6 days at 4°C in 1.5 mL microcentrifuge tubes.

For reticulocyte quantification 1 μL of blood was incubated with 2.5 nM of MitoTracker Deep Red FM (ThermoFisher) for 30 minutes (min) at 37°C. After washing with FACS buffer, samples were stained with antibodies against CD45, CD41, and CD71 for 30 min at 4°C (*Online Supplementary Table S1*).[13] After washing with FACS buffer, cells were analyzed using an Attune NxT flow cytometer (ThermoFisher). CD41$^+$ platelets and CD45$^+$ white blood cells were excluded and RBC were identified using a 405 nm filter (i.e., no wash no lyse filter, ThermoFisher)[13]. RBC were distinguished by CD71 expression: CD71$^+$ reticulocytes and CD71$^-$ mature RBC. Data were analyzed with FlowJo software (BD Biosciences).

Red blood cell transfusion, post-transfusion recovery, and red blood cell alloantibody detection

Stored RBC units were spiked 1:5 with fresh biotinylated RBC units before transfusion (200 uL) into recipients, as described.[26] Blood samples were collected via tail puncture and post-transfusion recovery (PTR) was measured as the ratio of biotin-positive RBC to GFP-positive RBC circulating 24 hours-post transfusion relative to the same ratio in the spiked blood unit. Sera were collected from recipients and total HOD alloantibodies (IgM + IgG + IgA) were quantified by flow crossmatch.[27]

Hematocrit and hemoglobin

Whole blood was collected from the submandibular vein into microcentrifuge tubes containing EDTA. Hematocrit was determined following micro-hematocrit capillary tube centrifugation. Hemoglobin was calculated spectrophotometrically (540 nm) using Drabkin's reagent.

Cytokine production and splenic erythrophagocytosis

Recipients were transfused with 6-day stored PHZ, stored control, or freshly collected GFP RBC units (350 μL). Plasma collected 2 hours post-transfusion was diluted 1:1 in duplicate. Cytokines were quantified by a fluorescence-encoded bead-based multiplex assay (LEGENDplex Mouse Anti-Virus Response Panel, BioLegend), per manufacturer's instructions. Spleens were processed into single cells[27] and splenocytes were stained with antibodies to delineate leukocyte subsets (*Online Supplementary Table S1*).[8,9,28,29] Results were acquired on an Attune NxT flow cytometer (ThermoFisher) and data analyzed with FlowJo software. Cell images were acquired on an Amnis ImageStreamX MkII cytometer (Luminex); 20,000 CD19+ B cells per file were recorded using INSPIRE software at 60x magnification. Analysis was performed using IDEAS 6.3 software.

Ultra-high-pressure liquid chromatography-mass spectrometry metabolomics

Metabolomics analyses were performed using a Vanquish ultra-high-pressure liquid chromatography (UHPLC) coupled online to a Q Exactive mass spectrometer (ThermoFisher, Bremen, Germany), as described.[30] Data were analyzed using Maven (Princeton University) and Compound Discoverer 2.1 (ThermoFisher). Graphs and statistical analyses were prepared with MetaboAnalyst 5.0.[31]

Protein digestion

Protein pellets from RBC units were digested, as described.[32]

Nano ultra-high-pressure liquid chromatography-tandem mass spectrometry proteomics

Sample processing and data collection were performed as described.[32]

Statistical analysis

A repeated measures two-way ANOVA with Sidak's multiple comparisons test was utilized for analysis of alloantibody production over time. For comparison of three or more groups, a one-way ANOVA with Tukey's multiple comparisons post-test was utilized. An unpaired *t* test was used to compare two groups; $P<0.05$ was considered significant. Analyses were performed using GraphPad Prism.

Results

Transfusion of stored reticulocyte-rich blood units enhances red blood cell alloimmunization

To test whether reticulocytes in RBC units modulate alloimmunization, RBC alloantibody production was evaluated in mice transfused with allogeneic RBC units containing elevated reticulocyte counts (experimental design in Figure 1A). In order to generate RBC units enriched in reticulocytes, reticulocytosis was induced by PHZ, which damages RBC, leading to hemolytic anemia followed by stress-induced reticulocytosis.[33] A PHZ dose titration revealed that CD71+ reticulocyte frequency peaked in peripheral blood 3-4 days post treatment, with an optimal dose of 50 mg/kg (*Online Supplementary Figure S1*). Thus, HOD RBC donor mice, which express an RBC-specific HOD alloantigen, were treated with PHZ or saline control to generate RBC units. Compared to control, reticulocyte-rich RBC units had higher reticulocyte levels (2% *vs.* 50%, $P<0.0001$; Figure 1B) and the reticulocytes had an increased frequency of mitochondria positivity (51% *vs.* 64%; $P<0.0001$; Figure 1C), as determined using Mito-Tracker dye. Allogeneic HOD RBC units were refrigerator-stored 6 days before transfusion into wild-type B6 animals; sera were collected weekly and assessed for anti-HOD alloantibodies by flow crossmatch. Transfusion of stored reticulocyte-rich RBC units (designated as "reticulocytes") induced significantly higher HOD alloantibody levels, compared to stored saline control RBC ("control") ($P<0.001$; Figure 1D), with an >800-fold difference in RBC alloantibody levels in recipients transfused with reticulocyte-rich RBC units, compared to control (Figure 1E). Additionally, all recipients transfused with reticulocyte-rich RBC units had detectable alloantibodies, compared to some animals in the control transfusion group that did not respond. In order to test whether the enhanced RBC alloimmunization was due to the method of inducing reticulocytosis, RBC units with higher reticulocyte frequencies were also generated using an iron-deficient/iron dextran injection mouse model. As with PHZ-induced reticulocytosis, transfusing reticulocyte-rich RBC units from iron-deficient/iron dextran injection donors induced significantly higher anti-HOD alloantibody levels, compared to controls (*Online Supplementary Figure S2*). Together, these data demonstrate that transfusing reticulocyte-rich RBC units induces higher RBC alloimmunization rates and alloantibody levels.

Transfusing stored reticulocyte-rich red blood cell units enhances proinflammatory cytokine production

In order to test whether transfusing reticulocyte-rich RBC units elicited an inflammatory response, plasma was collected 2 hours post-transfusion and assayed for cytokines. Transfusion of reticulocyte-rich RBC units led to significant increases in MCP-1, CXCL1, CXCL10, and IFN-γ levels,

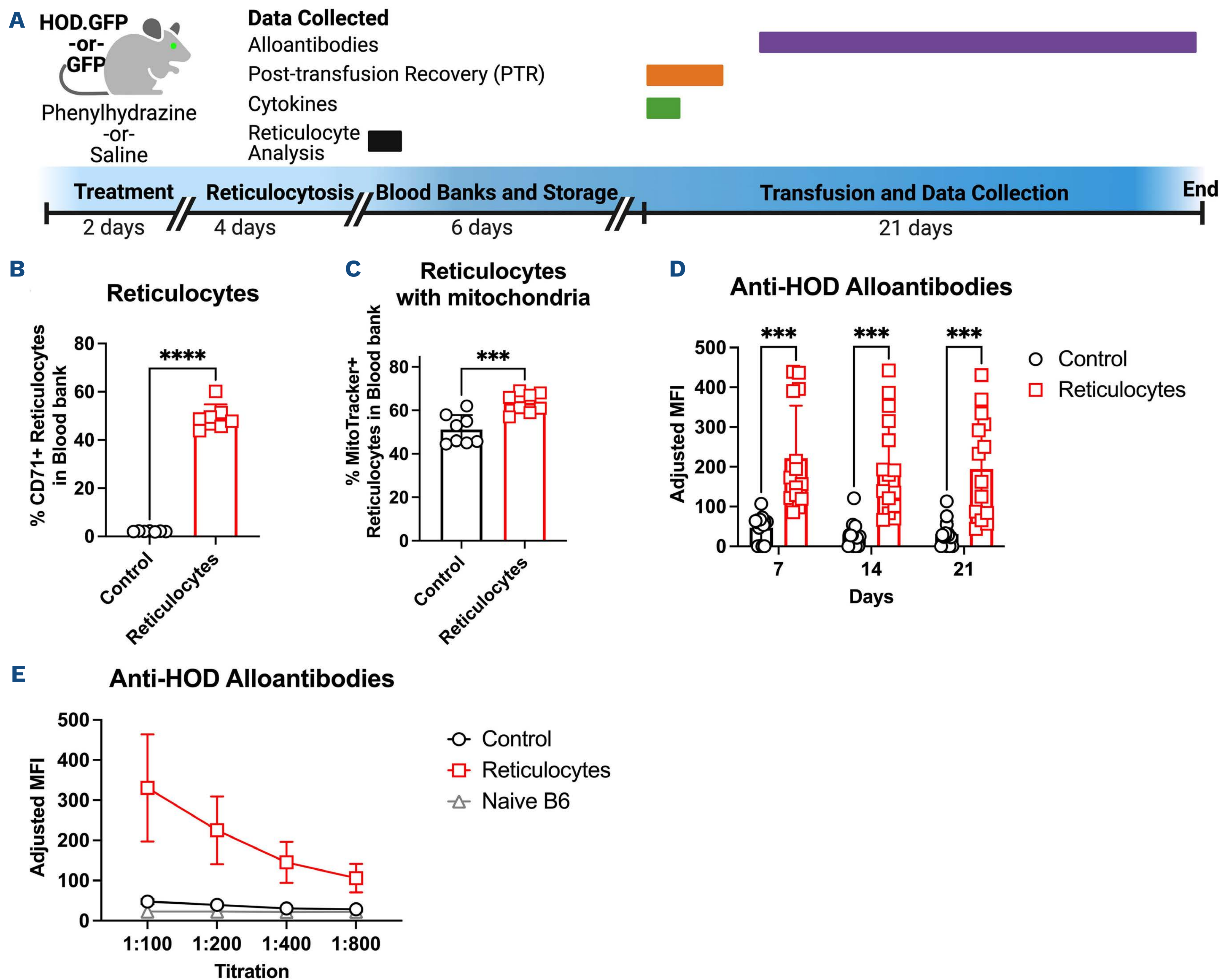

Figure 1. Transfusing refrigerator-stored reticulocyte-rich blood units enhanced red blood cell alloimmunization. (A) General experimental timeline. HOD mice received 2 intraperitoneal (i.p.) injections of phenylhydrazine (PHZ) (50 mg/kg, referred to as "Reticulocytes") or saline control (referred to as "Control") spaced 1 day apart. Four days after treatment, whole blood was collected by cardiac puncture into 14% CPDA-1, leuko-reduced, packed to a 60% hematocrit, and stored for 6 days at 4°C before transfusion (200 uL). For some experiments, on the day of transfusion, fresh red blood cells (RBC) were collected from biotinylated or GFP mice as an additional control. (B) The percent reticulocytes and (C) the percent of mitochondria-positive reticulocytes generated following PHZ and saline treatment. (D) Post-transfusion sera were analyzed for total anti-HOD RBC alloantibodies by flow crossmatch. (E) Sera collected at 14 days post transfusion were titrated. Naïve B6 sera were run in triplicate to establish the background antibody level. Data are cumulative from 3 independent experiments with 5 mice/group. Data with 2 groups were analyzed with an unpaired *t* test whereas 3 groups were analyzed by a repeated measures one-way ANOVA with Sidak's multiple comparisons test and ***$P<0.001$, ****$P<0.0001$. Timeline figure created with BioRender.

compared to control (Figure 2). No significant differences were noted in plasma IL-6, CCL5, IL-12, TNF-α, GM-CSF, IFN-α, IFN-β, IL-10, or IL-1β levels (*data not shown*). Together, these data suggest that transfusing reticulocytes elicited a strong, albeit selective, inflammatory response, characterized by significant chemokine production.

Reticulocyte-rich red blood cell units have elevated levels of mitochondrial metabolites and proteins

Untargeted and targeted metabolomics analyses using UHPLC-MS were performed on 6-day-stored reticulocyte-rich and control RBC units; detailed results are in the *Online Supplementary Table S2*. The metabolic phenotypes of reticulocyte-rich blood differed substantially from controls. Hierarchical clustering analysis (Figure 3A) highlighted significant associations of reticulocytes with metabolic pathway differences in lipid (fatty acid, phospholipid, and sphingolipid), amino acid, and purine metabolism. Volcano plots (*Online Supplementary Figure S3*) from targeted metabolomics analysis highlighted the top metabolites that increased (red) or decreased (blue) in reticulocyte-rich RBC units as compared to controls. Path-

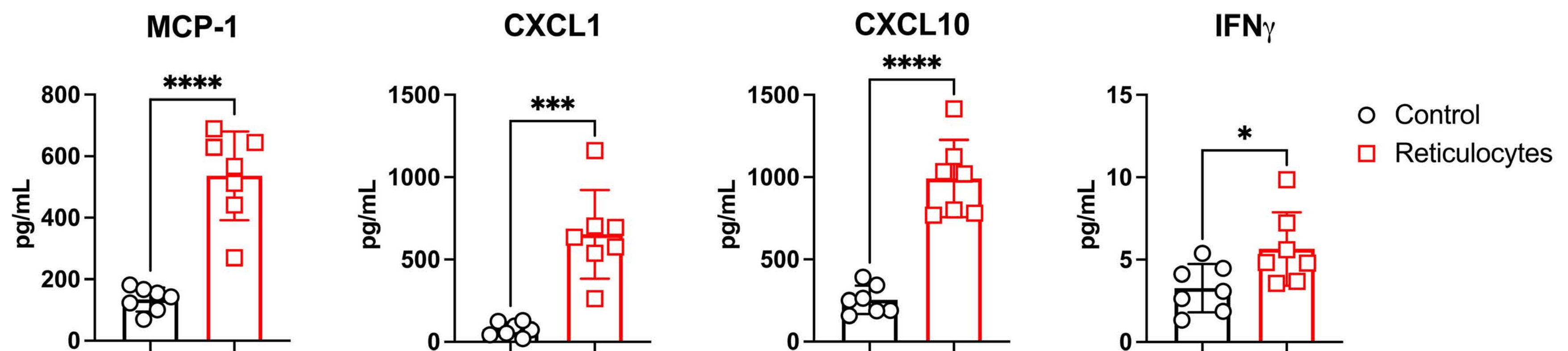

Figure 2. Induction of inflammatory cytokines after transfusing refrigerator-stored reticulocyte-rich red blood cell units. Reticulocyte-rich and control red blood cell (RBC) units were generated (as in Figure 1) using whole blood collected from GFP animals. RBC units were stored for 6 days and transfused into wild-type B6 animals. Plasma was collected 2 hours post-transfusion and analyzed for cytokines. Data shown are cumulative of 2 independent experiments with 3-4 mice/group. Data were analyzed with an unpaired t-test; *****P*<0.0001, ****P*<0.001, **P*<0.05.

way analysis of untargeted metabolomics data (Figure 3B) revealed altered regulation of metabolic pathways suggesting active mitochondrial function (i.e., β oxidation, tricarboxylic acid cycle [TCA], urea cycle, malate-aspartate shuttle, and glycerol-3-phosphate shuttle) and mitochondrial synthesis (cardiolipin synthesis).

Proteomics analyses were also performed on 6-day-stored reticulocyte-rich and control RBC units. Hierarchical clustering analysis (Figure 3C) highlighted significant associations of reticulocytes with proteins of mitochondrial origin upon gene ontology enrichment analysis for cell localization, with the most differentially expressed proteins involved in mitochondrial fatty acid metabolism, the TCA cycle, electron transport chain, ATP synthesis, mitochondrial structure, and mitochondrial oxidative stress (Figure 3D).

Stored reticulocyte-rich red blood cell units have reduced post-transfusion recovery and are preferentially ingested by splenic B cells

Based on enhanced cytokine production by, and the detectable mitochondrial byproducts in, reticulocyte-rich RBC units, we hypothesized that clearance of transfused RBC from the circulation would be accelerated. In order to test this, recipients were transfused with reticulocyte-rich or control RBC units and PTR (i.e., the percentage of transfused RBC remaining in the circulation for 24 hours) determined. Reticulocyte-rich RBC units had significantly reduced PTR at 24 hours, compared to control (*P*<0.0001; Figure 4A). To elucidate which cells were responsible for RBC clearance, reticulocyte-rich and control RBC units were generated using GFP mouse donors. Recipient B6 mice were then transfused with stored reticulocyte-rich, stored control, or freshly collected GFP RBC units and spleens harvested from recipients 2 hours later. Total splenocyte numbers were calculated and cells were stained with antibodies to identify antigen presenting cell (APC) subsets. For analysis, T cells and RBC (or cells with RBC attached to their surface) were excluded from total live splenocytes by excluding those positive for Thy1.2 and/or Ter119; GFP fluorescence provided an indirect measure of RBC consumption.[8,9,29] Enumerating leukocytes revealed a significant decrease in total cell numbers and in APC subsets, including F4/80$^+$ macrophages, CD8$^+$ DC, and CD19$^+$ B cells, in recipients transfused with reticulocyte-rich RBC units, compared to stored and fresh control RBC units (Figure 4B, C). No significant numerical differences in CD11b$^+$ DC were noted. Evaluating leukocytes for GFP fluorescence showed a significant increase in the frequency of GFP$^+$ cells, suggesting that transfused reticulocyte-rich RBC units were being consumed by more leukocytes, as compared to controls (*P*<0.0001; Figure 4D). Individual APC subsets were interrogated for GFP fluorescence to determine which participated in erythrophagocytosis. As previously reported,[34] transfusing stored control RBC units induced increased RBC consumption by macrophages, CD8$^+$ DC, and CD11b$^+$ DC, compared to fresh GFP control RBC (Figure 4E). However, as compared to stored control RBC units, RBC consumption from reticulocyte-rich units was significantly increased in CD8$^+$ and CD11b$^+$ DC; nonetheless, consumption by macrophages was not enhanced and was similar to fresh RBC controls. Unexpectedly, B cells markedly consumed RBC from transfused reticulocyte-rich units, compared to stored and fresh controls. These data demonstrate that transfusing reticulocyte-rich RBC units leads to significant changes in splenocyte cell subset composition and RBC consumption patterns. The most striking finding was that >20% of B cells had detectable GFP fluorescence, suggesting that a unique RBC consumption pattern may be associated with stored reticulocyte clearance.

In order to elucidate which splenic B cell subsets were involved in clearing reticulocyte-rich RBC units, B cells were gated into follicular, marginal zone, and B1 B-cell subsets (gating strategy in the *Online Supplementary Figure S4*).[28] Low levels of erythrophagocytosis by B-cell subsets were observed after transfusing stored or fresh control RBC units (Figure 5A). In contrast, there was a marked increase

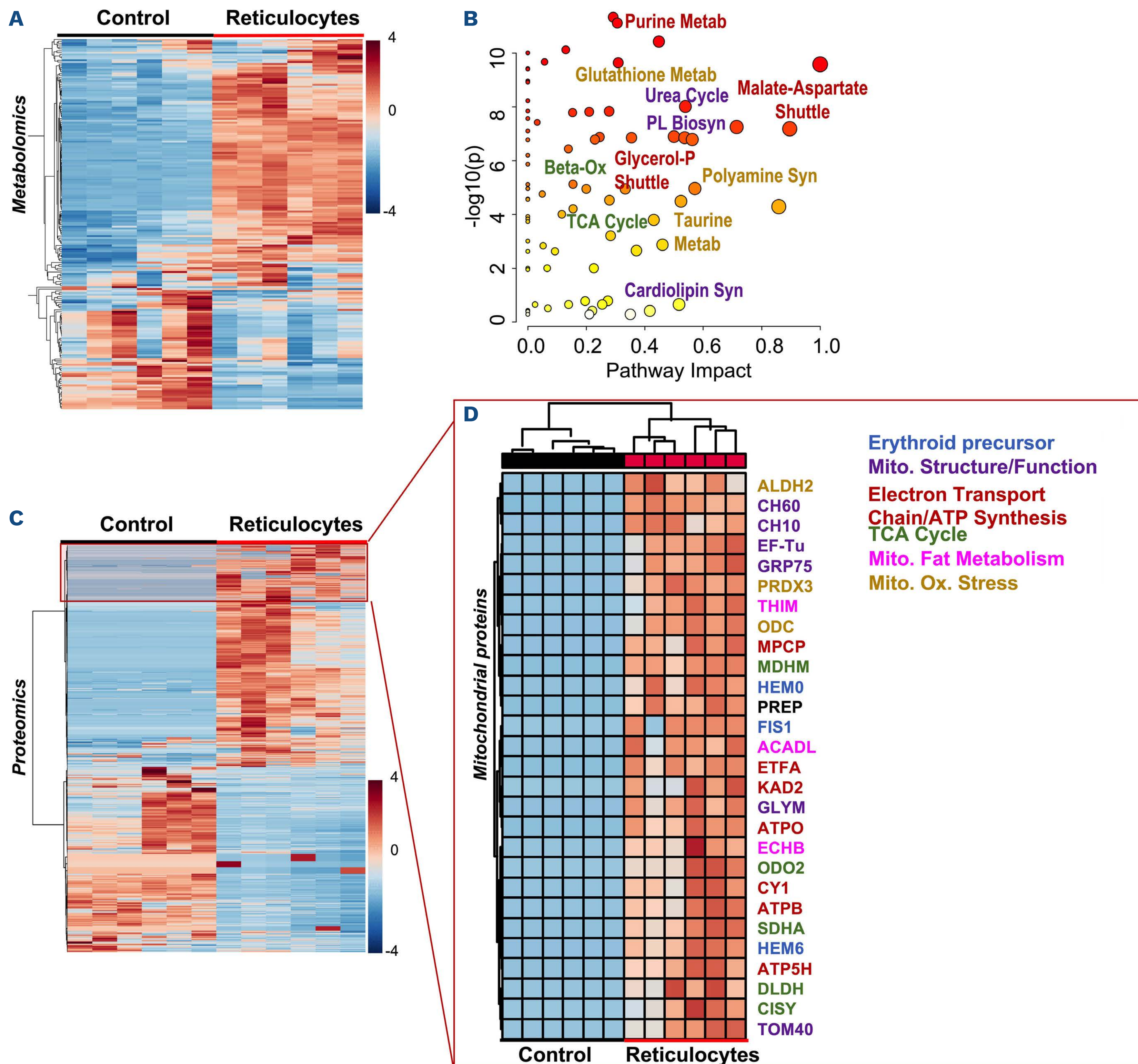

Figure 3. Omics analysis revealed mitochondrial metabolites and proteins in reticulocyte-rich red blood cell units. Aliquots of 6-day-stored reticulocyte and control red blood cell (RBC) units were subjected to omics analysis. (A) Hierarchical clustering analysis highlighting significant metabolic changes between control and reticulocyte-rich RBC units. (B) Pathway analysis of untargeted metabolomics data. (C) Hierarchical clustering of significantly different proteins between control and reticulocyte-rich RBC units. In (D), zoom in on a subset of mitochondrial proteins (by gene ontology of cell localization) whose levels are higher in reticulocyte-rich RBC units, compared to controls.

in GFP⁺ B cells following transfusion with reticulocyte-rich units. The most prominent signal was with innate B1 B cells, with approximately 45% positive for GFP. In order to confirm whether B cells actually internalized RBC, samples were analyzed with an imaging cytometer. With reticulocyte-rich samples, three GFP expression patterns emerged: diffuse GFP signal within CD19⁺ staining, punctate GFP signal along the cell membrane, and clusters of GFP signal within the B cell (Figure 5B, top; *Online Supplementary Figure S5*). In contrast, in controls, only punctate GFP signal along the B-cell membrane was detectable, along with a few cells that had RBC particulates attached to the B-cell surface (Figure 5B, bottom). In samples from the fresh control RBC transfusion group, very few GFP⁺ B cells were detected and most positive events reflected RBC particulates attached outside the B cell, as indicated by brightfield images (*Online Supplementary Figure S5*); this was consistent with the fre-

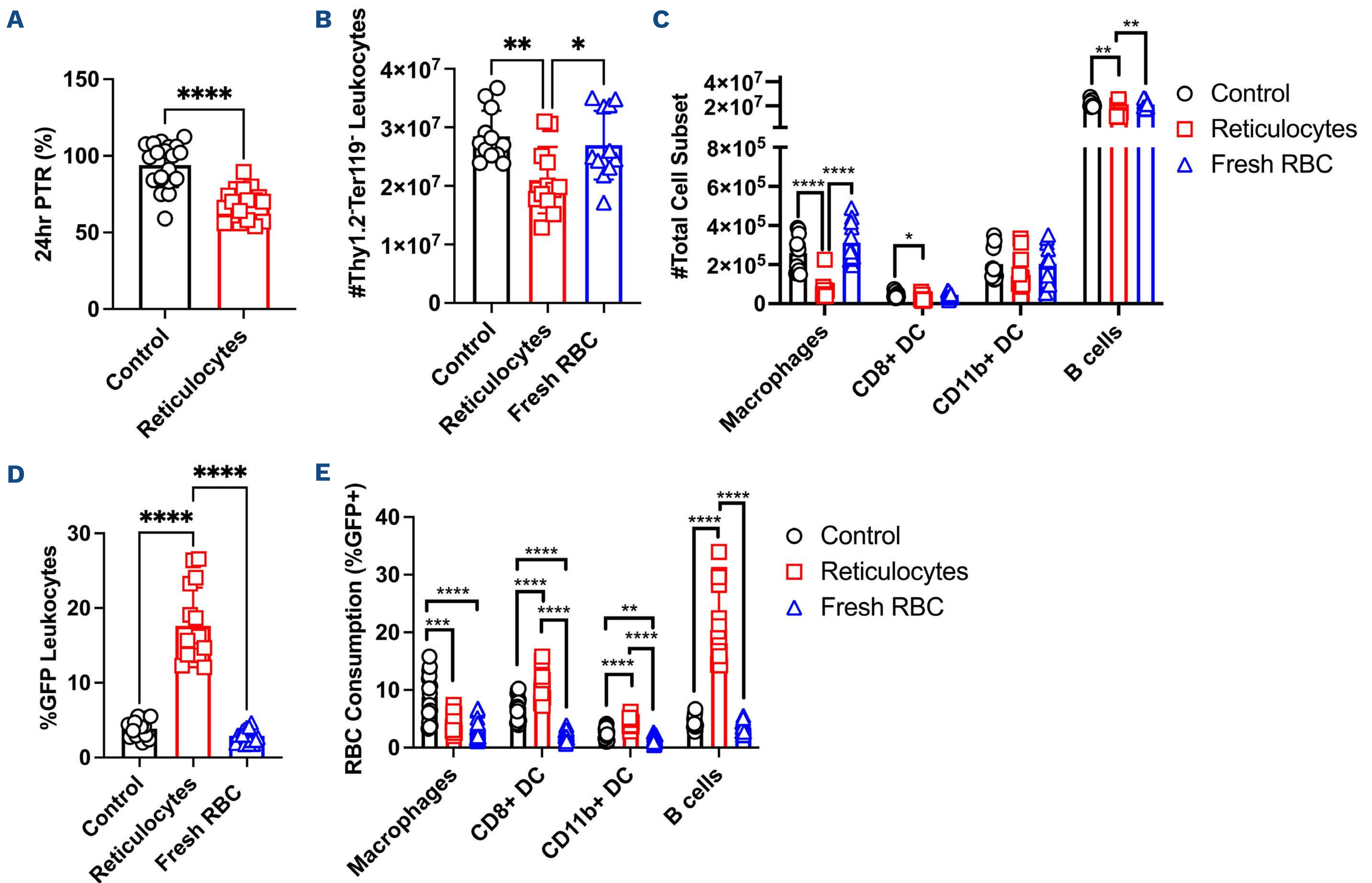

Figure 4. Transfusion with refrigerator-stored reticulocyte-rich red blood cell units led to increased red blood cell consumption. In order to determine post-transfusion recovery (PTR), reticulocyte-rich and control red blood cell (RBC) units were generated from GFP mice. After 6 days of storage, RBC units were spiked with freshly-biotinylated freshly-obtained RBC (as a tracer) and 350 μL were transfused into wild-type B6 recipients. (A) Whole blood was sampled 24 hours later and the frequency of GFP$^+$ RBC was normalized to biotinylated RBC to determine PTR. In order to evaluate cellular sites of RBC consumption, B6 recipients were transfused with 350 μL of freshly collected GFP$^+$ RBC or 6-day-stored RBC units (i.e., reticulocyte or control). Spleens were collected 2 hours post-transfusion and splenocytes stained with antibodies to delineate APC subsets. GFP signal level was used as an indirect measure of erythrophagocytosis. The total number of cells per spleen were calculated for (B) Thy1.2$^-$Ter119$^-$ leukocytes and for (C) individual APC subsets. The frequencies of GFP$^+$ (D) Thy1.2$^-$Ter119$^-$ leukocytes and (E) individual APC subsets were determined. The following phenotypes were used to delineate APC subsets: macrophages: CD11c$^{-/lo}$CD11b$^{-/lo}$F4/80$^+$; CD8$^+$ dendritic cells (DC): CD11c^{hi}CD8$^+$CD11b$^-$; CD11b$^+$ DC: CD11c^{hi}CD11b$^+$CD8$^-$; and B cells: CD19$^+$. Data are aggregated from at least 3 independent experiments with 3-4 mice/group. Data were analyzed with an unpaired *t* test for 2 groups or one-way ANOVA with Tukey's multiple comparisons test; *****P*<0.0001, ****P*<0.001, ***P*<0.01, **P*<0.05.

quency of cells with GFP signal inside the cell membrane (Figure 5C).

Red blood cell alloimmunization is enhanced, even after shorter storage and fewer reticulocytes

Because refrigerator storage adversely affects RBC quality, and because reticulocyte counts are much higher in reticulocyte-rich RBC units generated from mice, as compared to volunteer human donors, we generated mouse RBC units with defined reticulocyte frequencies. HOD RBC units were refrigerator-stored for 1 day (equivalent to ~3 days of storage for human RBC units) and then transfused into recipients. Transfusing RBC units with as few as 5% reticulocytes induced significant alloantibody production, as compared to control (i.e., <2% reticulocytes) (Figure 6A). There was a significant direct correlation between reticulocyte frequency in the RBC donor unit and alloantibody production; this relationship was significant even at low reticulocyte frequencies. Lastly, in order to test whether B-cell subsets consumed RBC from 1-day-stored reticulocyte-rich units, GFP fluorescence of follicular, marginal zone, and B1 B-cell subsets was assessed; significant RBC consumption was observed in each of these subsets following reticulocyte-rich RBC transfusions, compared to controls (Figure 6B). Thus, B-cell consumption patterns were similar between 1- and 6-day-refrigerator-stored reticulocyte-rich RBC units. These findings were visually confirmed by imaging cytometry (Figure 6C; *Online Supplementary Figure 6S*). Col-

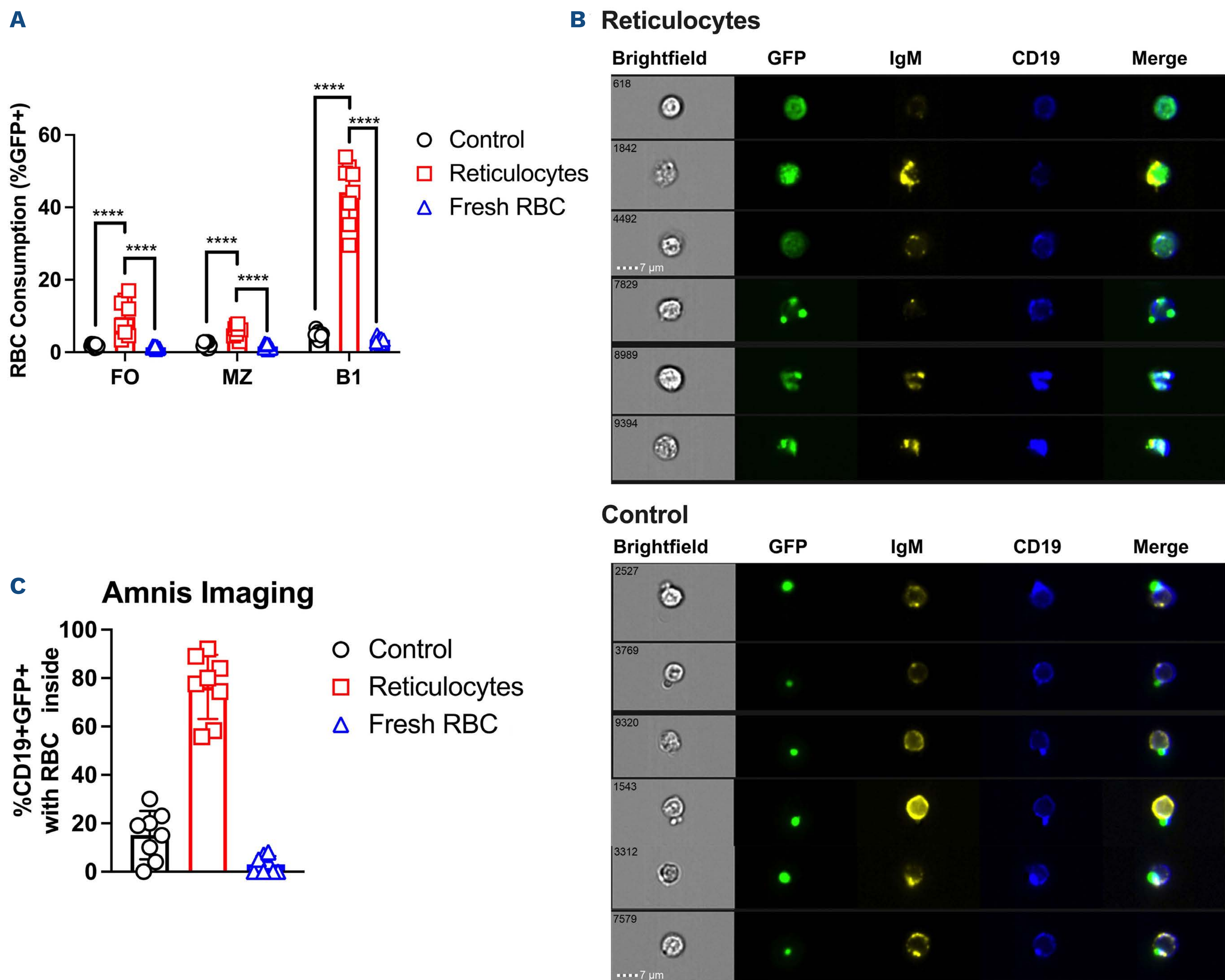

Figure 5. Multiple B-cell subsets consume stored reticulocytes. Recipients were transfused with 350 μL of freshly collected GFP+ red blood cells (RBC) or 6-day-stored RBC collected from phenylhydrazine (PHZ) or saline-treated mice. Spleens were collected 2 hours post-transfusion and splenocytes stained with antibodies to delineate APC subsets. GFP signal level was used as an indirect measure of erythrophagocytosis. (A) Total frequency of GFP+ cells for each B-cell subset was calculated by flow cytometry. (B) Imaging analysis of B cells from mice transfused with refrigerator-stored reticulocyte-rich (top) or stored saline (bottom) RBC units. Cell #4492 demonstrates RBC internalization by a B cell whereas #2527 represents an RBC adhered to the surface of a B cell. (C) Percentage of $CD19^+GFP^+$ cells with GFP^+ RBC located inside their cell membrane. The phenotypes used to delineate $CD19^+$ B-cell subsets were follicular (FO; $B220^+IgD^+IgMloCD23^+CD93^-$), marginal zone (MZ; $B220^+IgD^+IgM^+CD23^-CD93^-$), and B1 ($B220^{lo/-}$ $CD43^+$). In order to exclude RBC attached to the external B-cell surface, $Ter119^+$ cells were excluded from parent gates. Data are cumulative from 2 experiments with n=3-4 mice/group. Data were analyzed with one-way ANOVA with Tukey's multiple comparisons test; *****P*<0.0001.

lectively, these data show that even low reticulocyte counts make RBC units more immunogenic, and that prolonged refrigerator storage is not required for this enhanced alloimmunization.

TLR4 is not required for reticulocyte-mediated enhanced alloimmunization

Reticulocytes contain endogenous danger signals that can induce inflammation upon recognition by immune cells. Because stress-reticulocytes are more prone to hemolysis[20,21] (thereby releasing free heme[35]) and contain mitochondria (enveloped in cardiolipin),[36] we hypothesized that TLR4 was required for the observed enhanced alloimmune responses upon reticulocyte-rich transfusion. To that end, B6 and $TLR4^{-/-}$ recipients were transfused with reticulocyte-rich or control RBC units; no significant differences in anti-HOD alloantibodies were observed 14 days post-transfusion (Figure 7). Therefore, reticulocyte-mediated enhanced alloimmune responses do not require TLR4 signaling. These data also rule out the potential contribution of LPS contamination in RBC unit production.

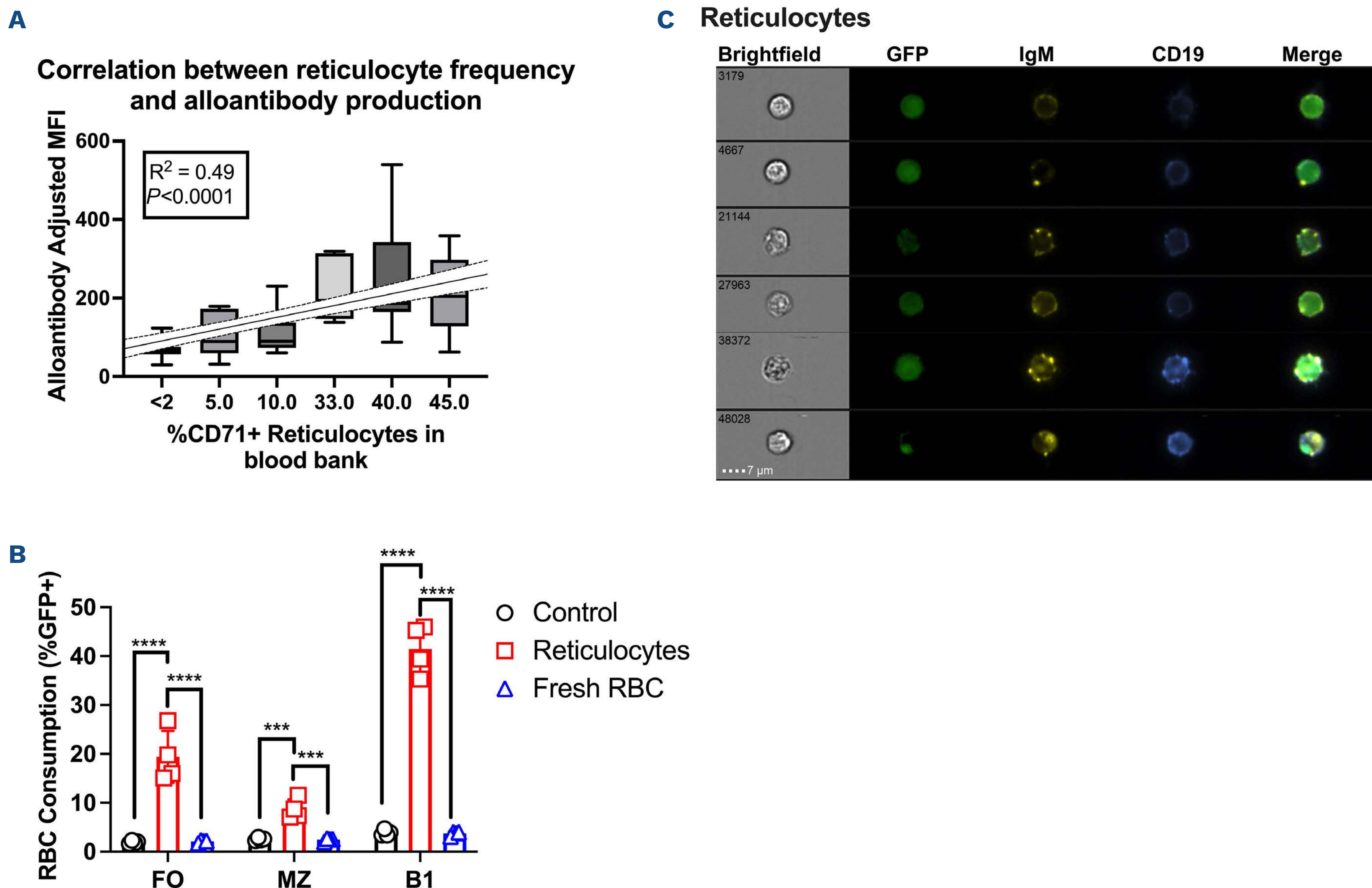

Figure 6. Red blood cell alloimmunization is stimulated, even with shorter storage duration and lower reticulocyte frequencies. Red blood cell (RBC) units with defined reticulocyte frequencies were generated by titrating whole blood from phenylhydrazine (PHZ)-treated animals into RBC collected from saline-treated animals. RBC units were leukoreduced and stored at 4°C for 1 day before transfusion into B6 recipient mice. (A) RBC alloantibodies were quantified by flow crossmatch at 14 days post-transfusion and alloantibody mean fluorescence intensity (MFI) was plotted against the RBC unit reticulocyte frequency. Data were analyzed with a one-way ANOVA and tested for linear trend. Data are cumulative of at least 3 independent experiments and each data point is 1 mouse, <2% (N=25), 5% (N=15), 10% (N=13), 33% (N=5), 40% (N=15), 45% (N=5). (B) Flow analysis revealed that multiple B-cell subsets consumed GFP^+ RBC from reticulocyte-rich RBC units and this was confirmed visually by (C) imaging analysis. For RBC consumption, data were analyzed with a one-way ANOVA with Tukey's multiple comparisons test; *****P*<0.0001, ****P*<0.001.

Discussion

These results demonstrate that increased reticulocyte count in a refrigerator-stored RBC unit can enhance transfusion-induced RBC alloimmune responses. Reticulocyte-mediated modulation of alloimmunization was dose-dependent, and was not an artifact of PHZ treatment as a similar effects were observed using reticulocytes generated from an iron deficiency/iron repletion model. Moreover, reticulocyte-enhanced alloimmunization was independent of refrigerator storage duration because transfusing reticulocyte-rich RBC units refrigerator stored for either 1 or 6 days enhanced alloimmunization rates and alloantibody levels. In order to explore underlying mechanisms, transfusion of reticulocyte-rich RBC units elicited pro inflammatory cytokines, and transfused RBC were cleared more rapidly, preferentially by splenic B cells. Lastly, we show that the observed reticulocyte-mediated enhanced alloimmune responses did not require TLR4 signaling; these data exclude the possibility of LPS contamination and rule out some potentially immunogenic reticulocyte-derived ligands, such as heme and cardiolipin. Together, these findings imply that reticulocytes in RBC units may be immunogenic and could enhance alloimmune responses, even in otherwise stable recipients; this finding may have implications for transfusions into all human patients, particularly those at highest risk for allo-immunization.

Approximately 70% of the blood supply in the USA derives from repeat volunteer blood donors.[37,38] Because blood donation typically removes approximately 10% of the total blood volume, compensatory stress erythropoiesis increases production of erythroid precursors and the premature release of reticulocytes; although the minimum inter-donation interval for blood donation in the US is 56 days, elevated reticulocyte counts (over baseline) are ob-

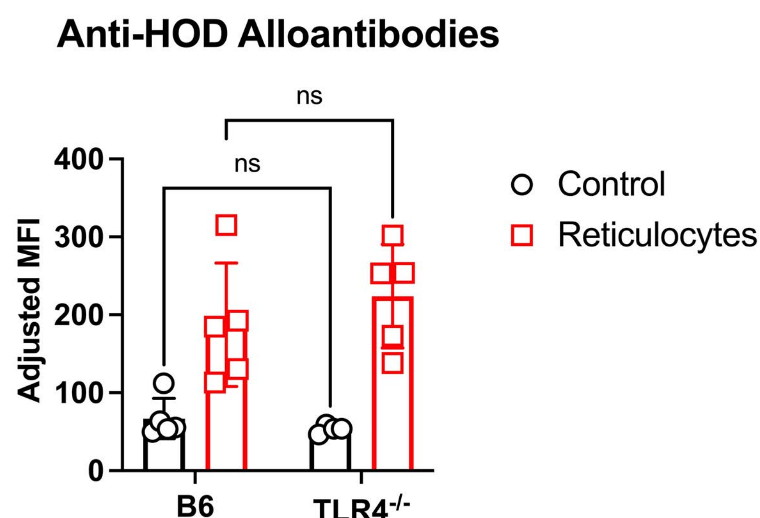

Figure 7. TLR4 is not required for reticulocyte-mediated enhanced red blood cell alloimmunization. Reticulocyte-rich and control red blood cell (RBC) units, generated from phenylhydrazine (PHZ)-treated HOD donor animals, were leuko-reduced and stored 1 day at 4°C before transfusion into wild-type B6 or TLR4$^{-/-}$ mice. Sera was collected 14 days post-transfusion and analyzed for total anti-HOD RBC alloantibodies by flow crossmatch. Data are from 1 experiment with 4-5 mice/group. Data were analyzed with a two-way ANOVA with Sidak's multiple comparisons test; ns: not significant.

served even after approximately 160 days after donation.[15,39] Thus, frequent donation by repeat blood donors can yield RBC units with higher reticulocyte counts. Additionally, current guidance for iron-deficient volunteers is to take iron supplements before donation, which also promote stress erythropoiesis.[15] Reticulocytes differ from their mature counterparts by: i) containing immune-enhancing organelles (e.g., mitochondria), lipids, and residual RNA; ii) expressing surface antigens that promote adherence (e.g., CD44, CD36); and iii) being larger and less deformable. Compared to steady-state reticulocytes, stress erythropoiesis-induced reticulocytes also contain significantly more organelles (e.g., mitochondria, ribosomes), express higher levels of surface molecules mediating adherence, and have shorter circulatory lifespans due to their size, decreased deformability, and susceptibility to shear stress.[18-23] Thus, reticulocytes, and especially stress-induced reticulocytes, contain and/or express immune-enhancing components.

Recipient inflammation is associated with higher alloimmunization rates and alloantibody levels.[4,5] Because RBC alloimmunization correlates with type I IFN levels, and because stress-induced reticulocytes contain ligands that could stimulate type I IFN production (e.g., mitochondrial DNA[13,14]), we measured plasma IFN-α and IFN-β. Unexpectedly, no significant levels were detectable. This could be due to timing, as plasma was collected 2 hours post-transfusion, or location (e.g., production localized to the spleen); additionally, it could indicate that type I IFN are not required for reticulocyte-enhanced alloimmunization. Nonetheless, transfusion of reticulocyte-rich units elicited high levels of proinflammatory cytokines and chemokines. Other potentially immunogenic reticulocyte components include mitochondria-derived cardiolipin and heme, both of which are TLR4 ligands. However, transfusing reticulocyte-rich RBC units into TLR4$^{-/-}$ and control recipients led to similar alloantibody levels (Figure 7), demonstrating TLR4 is dispensable for these enhanced alloimmune responses. Future studies will identify which signaling pathways are required for reticulocyte-mediated enhanced alloimmunity.

In a departure from typical RBC clearance patterns,[8] B cells preferentially consumed RBC derived from reticulocyte-rich units. Macrophages are typically responsible for steady-state removal of senescent and/or damaged RBC.[8,34] In murine models of RBC alloimmunization, with transfusion of stored RBC or in the context of inflammation, macrophages and DC subsets (CD11b^{+} and CD8^{+}) play critical roles in initiating alloimmune responses.[9,34] Although B-cell consumption of transfused RBC is, indeed, a prerequisite for alloantibody production, very few B cells are typically observed in this process. In contrast, B cells play a major role clearing transfused RBC from reticulocyte-rich units. Because of the high frequency of follicular and marginal zone B cells co-localizing with RBC, it is unlikely that RBC phagocytosis is B-cell receptor (BCR)-mediated; however, B1 B cells express polyreactive BCR that bind many autoantigens, including phosphatidylserine (PS), which is expressed at high levels on stress-induced reticulocytes.[40,41] As such, future studies will strive to identify which RBC characteristics of RBC (e.g., PS expression, mitochondrial content) are required for their enhanced consumption by B cells and subsequent alloantibody production. Additionally, innate B1 B cells can participate in BCR-independent endocytosis,[42] although the underlying mechanisms are not yet defined. As all B cells express complement receptors and Fc receptors,[43,44] these pathways may be involved in erythrophagocytosis. Elucidating the key pathways required for B-cell activation following their consumption of RBC in this setting will be an important focus of future experiments.

As anemia of many etiologies induce stress erythropoiesis, one potential limitation of these studies is that various stresses may produce differences in the resulting reticulocytes. Thus, it is possible that reticulocyte-containing RBC units generated from repetitively phlebotomized animals may not enhance RBC alloimmunization. In order to address this concern, the current studies utilized two different models of anemia—chemically-induced hemolytic anemia and iron deficiency anemia followed by iron repletion—to induce reticulocytosis. Transfusing RBC units generated with either model enhanced alloimmunization rates and alloantibody levels, as compared to controls. Another limitation is the inability to distinguish steady-state and stress-induced reticulocytes to assess their relative contributions to RBC alloimmunization; we are optimizing approaches to define a distinct phenotype for

stress-induced reticulocytes, which would be essential for screening RBC donor units and attributing specific functional outcomes in response to post-transfusion clearance. Although most studies presented herein utilized artificially high reticulocyte frequencies in donor RBC units for initial phenomenological studies, clinically relevant enhanced alloimmune responses were observed with as few as 5% reticulocytes (Figure 6A); it is noteworthy that the average reticulocyte frequency in unmanipulated animals in our studies is approximately 2% (Figure 6A), which is approximately twice the average observed in humans; this is likely due to the shorter lifespan of RBC in mice (~45 days) compared to humans (~100-120 days).[45] Thus, while these findings may be clinically relevant to human transfusion, studying reticulocyte-directed immune responses is biologically relevant to many other systems (e.g., neocytolysis,[46] malaria,[47] production of *in vitro* universal donor RBC[48] etc.).

RBC transfusion is an essential therapy in numerous diseases and is the most common inpatient therapeutic procedure requiring consent, with approximately 11 million transfusions per year in the US.[49] Although RBC transfusions can be lifesaving, some patients develop alloantibodies against donor RBC blood group antigens. RBC alloimmunization is clinically significant because it can cause adverse events (e.g., hyperhemolysis) and pose barriers to future care (e.g., organ transplantation). In addition, alloantibody prevention and detection, and mitigation of adverse events, currently requires significant medical, financial, and human resources. As such, identifying risk factors that promote alloimmunization is a high priority and would allow for reallocation of resources to patients most at-risk and provide potential therapeutic interventions. Herein, we demonstrate that the reticulocyte count in donor RBC units can enhance RBC alloimmunization, suggesting that increased reticulocyte frequencies may make RBC units more immunogenic and screening for reticulocyte count and/or lengthening the time between donations for repeat blood donors may reduce alloimmunization events.

Disclosures

Although unrelated to this manuscript, KEH has a sponsored research agreement with Alpine Immune Sciences. SLS is a scientific advisory board member (Hemanext, Inc. and Alcor, Inc.), consultant (Tioma, Inc. and Team Conveyer Intellectual Properties), executive director for Worldwide Initiative for Rh Disease Eradication (WIRhE), and CEO for Ferrous Wheel Consultants. All other authors have no conflicts of interest to disclose.

Contributions

TAT, EAH, SLS and KEH designed the studies and experiments. AQ, CYK, DEG, AM, MT, and FDZ collected and analyzed data from murine experiments. MD and AD performed the metabolomics and proteomics analyses. All authors were involved in the interpretation of data. TAT, EAH, SLS, and KEH wrote the manuscript. All authors contributed to the manuscript and approved the submitted version.

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Data-sharing statement

Original data and protocols are available to other investigators upon request by contacting the corresponding author.

References

1. Tormey CA, Hendrickson JE. Transfusion-related red blood cell alloantibodies: induction and consequences. Blood. 2019;133(17):1821-1830.
2. Roubinian NH, Reese SE, Qiao H, et al. Donor genetic and nongenetic factors affecting red blood cell transfusion effectiveness. JCI Insight. 2022;7(1):e152598.
3. Rapido F, Brittenham GM, Bandyopadhyay S, et al. Prolonged red cell storage before transfusion increases extravascular hemolysis. J Clin Invest. 2017;127(1):375-382.
4. Ryder AB, Zimring JC, Hendrickson JE. Factors influencing RBC alloimmunization: lessons learned from murine models. Trans Med Hemother. 2014;41(6):406-419.
5. Evers D, van der Bom JG, Tijmensen J, et al. Red cell alloimmunisation in patients with different types of infections. Br J Haematol. 2016;175(5):956-966.
6. Gibb DR, Liu J, Natarajan P, et al. Type I IFN Is Necessary and

sufficient for inflammation-induced red blood cell alloimmunization in mice. J Immunol. 2017;199(3):1041-1050.
7. Lee JY, Madany E, El Kadi N, et al. Type 1 interferon gene signature promotes RBC alloimmunization in a Lupus mouse model. Front Immunol. 2020;11:584254.
8. Richards AL, Hendrickson JE, Zimring JC, Hudson KE. Erythrophagocytosis by plasmacytoid dendritic cells and monocytes is enhanced during inflammation. Transfusion. 2016;56(4):905-916.
9. Richards AL, Sheldon K, Wu X, Gruber DR, Hudson KE. The role of the immunological synapse in differential dffects of APC subsets in alloimmunization to fresh, non-stored RBCs. Front Immunol. 2018;9:2200.
10. Rönnblom L, Eloranta ML. The interferon signature in autoimmune diseases. Curr Opin Rheumatol. 2013;25(2):248-253.
11. Kim M, Hwang S, Park K, Kim SY, Lee YK, Lee DS. Increased expression of interferon signaling genes in the bone marrow microenvironment of myelodysplastic syndromes. PLoS One. 2015;10(3):e0120602.
12. Madany E, Lee J, Halprin C, et al. Altered type 1 interferon responses in alloimmunized and nonalloimmunized patients with sickle cell disease. EJHaem. 2021;2(4):700-710.
13. Moriconi C, Dzieciatkowska M, Roy M, et al. Retention of functional mitochondria in mature red blood cells from patients with sickle cell disease. Br J Haematol. 2022;198(3):574-586.
14. Caielli S, Cardenas J, de Jesus AA, et al. Erythroid mitochondrial retention triggers myeloid-dependent type I interferon in human SLE. Cell. 2021;184(17):4464-4479.
15. Mast AE, Szabo A, Stone M, Cable RG, Spencer BR, Kiss JE. The benefits of iron supplementation following blood donation vary with baseline iron status. Am J Hematol. 2020;95(7):784-791.
16. Wong E, Rose M, Berliner N. Disorders of red blood cells. Cecil Essentials of Medicine, Tenth Edition. Elsevier. 2022:489-500.
17. Stryckmans PA, Cronkite EP, Giacomelli G, Schiffer LM, Schnappauf H. The maturation and fate of reticulocytes after in vitro labeling with tritiated amino acids. Blood. 1968;31(1):33-43.
18. Come SE, Shohet SB, Robinson SH. Surface remodelling of reticulocytes produced in response to erythroid stress. Nat New Biol. 1972;236(66):157-158.
19. Come SE, Shohet SB, Robinson SH. Surface remodeling vs. whole-cell hemolysis of reticulocytes produced with erythroid stimulation or iron deficiency anemia. Blood. 1974;44(6):817-830.
20. Noble NA, Xu Q-P, Hoge LL. Reticulocytes II: reexamination of the in vivo survival of stress reticulocytes. Blood. 1990;75(9):1877-1882.
21. Robinson SH, Tsong M. Hemolysis of "stress" reticulocytes: a source of erythropoietic bilirubin formation. J Clin Invest. 1970;49(5):1025-1034.
22. Carden MA, Fasano RM, Meier ER. Not all red cells sickle the same: contributions of the reticulocyte to disease pathology in sickle cell anemia. Blood Rev. 2020;40:100637.
23. Sawadogo D, Tolo-Dilkébié A, Sangaré M, Aguéhoundé N, Kassi H, Latte T. Influence of the clinical status on stress reticulocytes, CD36 and CD49d of SSFA 2 homozygous sickle cell patients followed in Abidjan. Adv Hematol. 2014;2014:273860.
24. Desmarets M, Cadwell CM, Peterson KR, Neades R, Zimring JC. Minor histocompatibility antigens on transfused leukoreduced units of red blood cells induce bone marrow transplant rejection in a mouse model. Blood. 2009;114(11):2315-2322.
25. Bandyopadhyay S, Brittenham GM, Francis RO, Zimring JC, Hod EA, Spitalnik SL. Iron-deficient erythropoiesis in blood donors and red blood cell recovery after transfusion: initial studies with a mouse model. Blood Trans. 2017;15(2):158-164.
26. Kim CY, Johnson H, Peltier S, et al. Deuterated linoleic acid attenuates the RBC storage lesion in a mouse model of poor RBC storage. Front Physiol. 2022;13:868578.
27. Qiu A, Miller A, Zotti FD, et al. FcγRIV is required for IgG2c mediated enhancement of RBC alloimmunization. Front Immunol. 2022;13:972723.
28. Richards AL, Howie HL, Kapp LM, Hendrickson JE, Zimring JC, Hudson KE. Innate B-1 B cells are not enriched in red blood cell autoimmune mice: importance of B cell receptor transgenic selection. Front Immunol. 2017;8:1366.
29. Richards AL, Qiu A, Zotti FD, et al. Autoantigen presentation by splenic dendritic cells is required for RBC-specific autoimmunity. Transfusion. 2021;61(1):225-235.
30. Nemkov T, Reisz JA, Gehrke S, Hansen KC, D'Alessandro A. High-throughput metabolomics: isocratic and gradient mass spectrometry-based methods. Methods Mol Biol. 2019;1978:13-26.
31. Pang Z, Chong J, Zhou G, et al. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. Nucleic Acids Res. 2021;49(W1):W388-W96.
32. Thomas T, Stefanoni D, Dzieciatkowska M, et al. Evidence of structural protein damage and membrane lipid remodeling in red blood cells from COVID-19 patients. J Proteome Res. 2020;19(11):4455-4469.
33. Shetlar MD, Hill HA. Reactions of hemoglobin with phenylhydrazine: a review of selected aspects. Environ Health Perspect. 1985;64:265-281.
34. Youssef L, Rebbaa A, Pampou S, et al. Increased erythrophagocytosis induces ferroptosis in red pulp macrophages in a mouse model of transfusion. Blood. 2018;131(23):2581-2593.
35. Janciauskiene S, Vijayan V, Immenschuh S. TLR4 signaling by heme and the role of heme-binding blood proteins. Front Immunol. 2020;11:1964.
36. Pizzuto M, Pelegrin P. Cardiolipin in immune signaling and cell death. Trends Cell Biol. 2020;30(11):892-903.
37. Whitaker B, Rajbhandary S, Kleinman S, Harris A, Kamani N. Trends in United States blood collection and transfusion: results from the 2013 AABB Blood Collection, Utilization, and Patient Blood Management Survey. Transfusion. 2016;56(9):2173-2183.
38. Kiss JE, Vassallo RR. How do we manage iron deficiency after blood donation? Br J Haematol. 2018;181(5):590-603.
39. Schotten N, Pasker-de Jong PCM, Moretti D, et al. The donation interval of 56 days requires extension to 180 days for whole blood donors to recover from changes in iron metabolism. Blood. 2016;128(17):2185-2188.
40. Hosseini H, Li Y, Kanellakis P, et al. Phosphatidylserine liposomes mimic apoptotic cells to attenuate atherosclerosis by expanding polyreactive IgM producing B1a lymphocytes. Cardiovasc Res. 2015;106(3):443-452.
41. Baumgarth N. How specific is too specific? B-cell responses to viral infections reveal the importance of breadth over depth. Immunol Rev. 2013;255(1):82-94.
42. Parra D, Rieger AM, Li J, et al. Pivotal advance: peritoneal cavity B-1 B cells have phagocytic and microbicidal capacities and present phagocytosed antigen to CD4+ T cells. J Leukoc Biol. 2012;91(4):525-536.
43. Liu J, Wang Y, Xiong E, et al. Role of the IgM Fc receptor in immunity and tolerance. Front Immunol. 2019;10:529.
44. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. Cell Res. 2010;20(1):34-50.
45. Krzyzanski W, Brier ME, Creed TM, Gaweda AE. Reticulocyte-

based estimation of red blood cell lifespan. Exp Hematol. 2013;41(9):817-822.
46. Mairbäurl H. Neocytolysis: How to get rid of the extra erythrocytes formed by stress erythropoiesis upon descent from high altitude. Front Physiol. 2018;9:345.
47. Lim C, Pereira L, Saliba KS, et al. Reticulocyte preference and stage development of Plasmodium vivax isolates. J Infect Dis. 2016;214(7):1081-1084.
48. Pellegrin S, Severn CE, Toye AM. Towards manufactured red blood cells for the treatment of inherited anemia. Haematologica. 2021;106(9):2304-2311.
49. Jones JM, Sapiano MRP, Mowla S, Bota D, Berger JJ, Basavaraju SV. Has the trend of declining blood transfusions in the United States ended? Findings of the 2019 National Blood Collection and Utilization Survey. Transfusion. 2021;61 (Supple 2)S1-10.