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Stored RBC Transfusions Leads to the Systemic Inflammatory Response Syndrome in Anemic Murine Neonates

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Abstract

Objective: RBC transfusions (RBCT) are life-saving treatment for premature and critically ill infants. However, the procedure has been associated with the development of systemic inflammatory response syndrome (SIRS) and potentially multiple organ dysfunction syndrome (MODS) in neonates. The present study aimed to investigate the mechanisms of RBCT-related SIRS in severely anemic murine neonates.

Methods: C57BL/6 (WT), TLR4$^{-/-}$ and myeloid-specific triggered myeloid receptor-1 (*trem1*)$^{-/-}$ mouse pups were studied in 4 groups (n=6 each): (1) naïve controls, (2) transfused control, (3) anemic (hematocrit 20–24%) and (4) anemic with RBC transfused using our established murine model of phlebotomy-induced anemia (PIA) and RBC transfusion. Plasma was measured for quantifying inflammatory cytokines (IFN-γ, IL-1β, TNF-α, IL-6, MIP-1α,MIP-1β, MIP2 and LIX) using a Luminex assay. *In vitro* studies included (i) sensitization by exposing the cells to a low level of lipopolysaccharide (LPS; (500 ng/ml) and (ii) *trem1*-siRNA transfection with/without plasma supernatant from stored RBC to assess the acute inflammatory response through *trem1* by qRT-PCR and immunoblotting.

Results: Anemic murine pups developed cytokine storm within 2 hours of receiving stored RBCs, which increased until 6h post-transfusion, as compared to non-anemic mice receiving stored RBCTs (“transfusion controls”), in a TLR4-independent fashion. Nonetheless, severely

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anemic pups had elevated circulating endotoxin levels, thereby sensitizing circulating monocytes to presynthesize proinflammatory cytokines (IFN-γ, IL-1β, TNF-α, IL-6, MIP-1α, MIP-1β, MIP2, LIX) and express trem1. Silencing trem1 expression in RAW264.7 cells mitigated both endotoxin-associated presynthesis of proinflammatory cytokines and the RBCT-induced release of inflammatory cytokines. Indeed, myeloid-specific *trem1*$^{-/-}$ murine pups had significantly reduced evidence of SIRS following RBCTs.

Conclusion: Severe anemia-associated low-grade inflammation sensitizes monocytes to enhance the synthesis of proinflammatory cytokines and *trem1*. In this setting, RBCTs further activate these monocytes, thereby inducing SIRS. Inhibiting trem1 in myeloid cells, including monocytes, alleviates the inflammatory response associated with the combined effects of anemia and RBCTs in murine neonates.

Keywords

Neonatal anemia; monocytes; proinflammatory cytokines; Systemic Inflammatory Response Syndrome; triggered myeloid receptor protein 1

Introduction

Anemia is nearly universal in preterm infants and is associated with increased morbidity and mortality worldwide[1–9]. Infant hematopoiesis is gradually activated over the first 6–8 weeks of life after an initial period of physiological suppression due to relative hyperoxia *ex utero*, which is exacerbated in preterm infants as the burden of hematopoietic signaling has not yet shifted from the liver to the more oxygen-responsive kidney. During this early neonatal period, impaired hematopoiesis in the setting of normal RBC turnover leads to progressive anemia. In sick and/or preterm infants, this phenomenon is amplified by phlebotomy losses inherent to intensive care, resulting in steeper anemia that overwhelms physiologic processes and frequently leads to the need for one or more RBC transfusions[2, 4, 9–11]. Preterm neonates comprise one of the most heavily transfused populations, as 50–94% of very low birth weight (VLBW) infants receive at least one RBCT during their stay in the neonatal intensive care unit (NICU)[7, 12–14]. RBCT is, therefore, commonly employed, although it carries potential risks[15–17]. Despite the risks, RBCTs are necessary and lifesaving in premature and critically ill infants experiencing severe anemia due to either physiologic or iatrogenic factors[17–19]. Stable critically ill children transfused with long-stored leukoreduced RBC units may be at increased risk of developing new or progressive Systemic Inflammatory Response Syndrome (SIRS)[15, 20–24]. However, this relationship is not yet mechanistically understood in terms of the underlying anemia and/or the RBCTs. The current study addresses this fundamental issue by aiming to understand the role of severe anemia in transfusion-associated SIRS, which is critical to identifying safe transfusion practices for these vulnerable neonates.

We recently showed that severe anemia was associated with increased intestinal permeability, as demonstrated by detecting endotoxin in the circulation[5], associated with increased monocyte infiltration in the hypoxic intestine. Subsequent RBCTs then activate these monocytes and cause a necrotizing enterocolitis (NEC)-like intestinal injury[6]. NEC characteristically follows an initial, early stage of the systemic inflammatory response,

followed by a “definitive” stage of localized peritonitis, and then a final, advanced stage of diffuse peritonitis[25, 26]. We hypothesized that anemia-associated “leaky gut” allows the entry of luminal bacteria into the circulation and sensitizes circulating monocytes with preformed proinflammatory cytokines. RBCTs induce acute release of these preformed proinflammatory cytokines, thereby causing SIRS. The current study was designed to investigate the early effects (i.e., within 6 hours) and mechanisms by which anemia and RBCTs cause acute inflammatory responses before the development of NEC-like injury (at 6 hours post-RBCT). Using our existing murine model of phlebotomy-induced anemia followed by RBCT[5, 6, 27], we now show that anemia induces circulating monocytes to synthesize proinflammatory cytokines (i.e., IFN-γ, IL-1β, TNF-α, IL6, MIP1α, MIP1β, MIP2 and LIX) along with expression of trem1, and the supernatant from transfusions of stored RBCs then triggers monocytes to release their pre-formed proinflammatory cytokines over the ensuing few hours. Targeting *trem1* mRNA expression in myeloid cells significantly mitigated this acute cytokine storm. Therefore, the results of this study suggest therapeutic strategies to reduce this anemia and RBCT-associated acute inflammatory response, which may be useful in preventing subsequent multi-organ dysfunction syndrome (MODS).

Materials and methods

Experimental design

Animal studies were performed after ethical approval from the Institutional Animal Care and Use Committees at Johns Hopkins University and the University of Nebraska Medical Center, in compliance with all relevant ethical regulations for animal testing and research. C57BL/6 and genetically modified mice from each litter, including both genders, were randomly assigned to four study groups: (a) naive control; (b) transfusion control; (c) anemic control; and (d) anemic-transfused mice as described previously[5, 6, 27, 28]. Briefly, Naive controls were maintained without intervention and anemic controls were subjected to facial vein phlebotomy to collect 20 μL/g of body weight of blood on postnatal (P) days P2, P4, P6, P8, and P10. Day P7 mice were inoculated with a clinical isolate of *Serratia marcescens* (10^4 CFU by gavage) grown in nutrient agar/broth (American Type Culture Collection, Manassas, VA). On day P11, naïve control and anemic control mice received an intravenous RBCT (20 mL per kg) into the retroorbital venous plexus, injected in two aliquots, one on each side, to provide transfusion controls and anemic-transfused mice, respectively. RBCs for transfusion were collected from adult allogenic FVB/NJ donors by retro-orbital bleeding into CPDA-1 solution (Sigma, St. Louis, MO; catalog #C4431; 6 parts blood:1 part CPDA-1), and leukoreduced using a sterile Acrodisc WBC syringe filter (Cytiva, Marlborough, MA; catalog #AP-4952). The leukoreduced blood was then centrifuged for 10 minutes at 200 x g and the supernatant was completely removed along with a top portion of the RBC layer (to remove platelets) to obtain a hematocrit of approximately 70% before storage at 4°C in the dark for 7 days. We used FVB/NJ mice for donors, since C57BL/6 recipients have no ”naturally occurring” antibodies that recognize FVB RBC antigens and FVB donors are allogenic, and are thus widely used for studying the effects of storage RBC and its transfusion[23]. Transfused RBCs were confirmed to be free of endotoxin contamination before use (limulus lysate assay; Thermo Fisher, Waltham, MA; catalog #88282) and measured free heme level according to the method previously described[6].

Hematocrit tests were performed at each phlebotomy and then on day P11; to this end, 20 μL blood was diluted 1:7 in Cellpack reagent (Sysmex America, Mundelein, IL; Cell Pack DCL # 300A) and analyzed in a Sysmex XN-1000TM veterinary hematology analyzer. Whole blood (30 μL) was collected from naïve controls and PIA groups at P11 before RBC transfusion and then at serial time points (2h, 4h, and 6h) post-transfusion by facial vein phlebotomy over the same mouse pups, placed into EDTA-coated tubes then centrifuged at 1400 x g for 10 minutes to obtained plasma. The plasma samples (approximately 20 μL) were immediately stored at −80°C for further analysis. The sample size for inflammatory responses was estimated at $\alpha = 0.05$ and 80% power (Lehmann's method for non-Gaussian data).

Genetically modified mice

B6(Cg)-Tlr4tm1.2Karp/J (stock #029015) and B6.129P2-Lyz2tm1(cre)lfo/J (stock #004781) were purchased from The Jackson Laboratory (Bar Harbor, ME) and used per vendor's guidelines. C57BL/6N-*Trem1tm1(KOMP)Vlcg*/Mmucd (MMRRC Stock # 050208)-UCD mice were purchased from the Mutant Mouse Resource & Research Center (MMRRC) at UC Davis (Davis, CA) and used per vendor's guidelines. To generate myeloid-specific knockouts of *trem1* (*trem1*$^{-/-}$), *trem1*^{flox} mice (C57BL/6N-*Trem1tm1(KOMP)Vlcg*/Mmucd; MMRRC Stock # 050208) were bred with transgenic mice expressing Cre under the control of the endogenous lysozyme 2 (Lyz2) promoter/enhancer elements B6.129P2-Lyz2tm1(cre)lfo/J (stock #004781), in which the Cre-mediated recombination results in deletion of the targeted gene in the myeloid cell lineage, including monocytes, mature macrophages and granulocytes.

Cytokine levels quantified by Luminex-multiplex array

Cytokine concentrations were measured by a magnetic bead-based multiplex assay using the Milliplex Map Mouse Cytokine/chemokine Magnetic Bead Panel -Premixed 32 plex – Immunology kit (Millipore Sigma; Burlington, MA; catalog #MCYTMAG-70K-PX32); it allows measurement of IFN-γ, IL-1β, TNF-α, IL6, MIP1α, MIP1β, MIP2, LIX and the assay was performed according to the manufacturer's instructions. Briefly, plasma samples were diluted 1:2 in sample dilution reagent and 50 μL was added to the respective wells of a 96-well plate. To each well, 50 μL of pre-mixed bead mixture was added, and the plate was incubated in the dark overnight in a cold room and shaken with a compact microplate shaker (Thermo Scientific, Waltham, MA; catalog #11676337) at 800 rpm. The plate was then placed on a magnetic plate separator (Magnetic Plate Separator, Luminex, Austin, Tx; catalog #CN-0269–01) and the supernatant was removed. The beads were then washed twice with 200 μL of washing buffer. Next, 25 μL of detector antibody solution was added to each well and the plate was incubated for 30 min, shaking at 800 rpm, at room temperature in the dark. Without removing the supernatant, 25 μL of Streptavidin—Phycoerythrin conjugate was added and incubated for 30 min and shaken at 800 rpm at room temperature in the dark. After removing the supernatant, the beads were washed twice. Next, 100 μL of sheath fluid was added to each well, the plate was incubated for 30 sec while shaking at 800 rpm at room temperature in the dark, and then immediately analyzed using a MAGPIX System (Millipore Sigma; Burlington, MA; catalog #40–071) and Luminex xPONENT software version 3.1

Build 971 (Luminex, Austin, Tx). In some studies, 50 μL of Raw264.7 cell culture media were analyzed as above to measure major cytokine expression (IFN-γ, IL-1β, TNF-α, IL6).

Intracellular cytokine staining and flow cytometry

Whole blood samples were incubated with RBC lysis buffer (eBioscience, San Diego, CA; catalog# 00–4333-57) for 10 min at RT for RBC lysis and after washing with PBS by centrifugation, the pellet was resuspended in cell staining buffer (BioLegend, San Diego, CA; catalog# 420201). Cell surface staining, followed by intracellular staining, was performed using Fix & Perm Cell Fixation & Permeabilization Kit (Abcam, Waltham, MA; catalog #ab185917) according to the manufacturer's protocol. Blood-derived cell suspension was subjected to surface staining with antibodies (all from BioLegend, San Diego, CA) against CD45 (dilution 1:25; clone #30-F11; catalog# 103106), CD11b (dilution 1:25; clone #M1/70; catalog# 101226), Ly6C (dilution 1:25; clone #HK1.4; catalog# 128036), F4/80 (dilution 1:25; clone #BM8; catalog# 123132), trem1 (dilution 1:25; clone # 174031; catalog# 747899). After incubation on ice for 30 min, cells were washed with cell staining buffer (BioLegend; catalog# 420201), and the pellet resuspended in 100 μL of Fixation Medium and incubated for 15 min. 5 mL of cell staining buffer was then added and cells were washed to obtain the pellet. Next, 100 μL permeabilization medium containing antibodies for major cytokines (IFN-γ (dilution 1:25; clone; XMG12, catalog# 505808), IL-1β (dilution 1:25; clone; CRM56; catalog# 12701841), TNF-α (dilution 1:25; clone; MP6-XT22, catalog# 506313), and IL-6 (dilution 1:25; clone; MP5–20F3, catalog# 46–7061-82) were added and incubated for 15 min on ice. Cells were washed again with cell staining buffer and data were acquired on a BD LSR-II flow cytometer and analyzed using the FlowJo version 10.5.3 software package (Becton Dickinson, Franklin Lakes, NJ).

Non-transferrin bound iron (NTBI) assay

Plasma NTBI was measured by a nitrilotriacetic acid (Sigma-Aldrich, St. Louis, MO; catalog #N9877) ultrafiltration assay[23, 29, 30]

Cell culture studies

RAW264.7 cells (ATCC, Manassas, VA; catalog #TIB-71), a murine macrophage cell line purchased from the American Type Culture Collection (ATCC, Manassas, VA), were grown in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) with 10% fetal calf serum. In some experiments, these cells were treated for different time intervals with different concentrations (0.1–100μg/ml) of *Escherichia coli* 0111:B4 lipopolysaccharide (Sigma, St. Louis, MO) or with the supernatant from stored RBCs (which were used for transfusion).

Plasmids and transfection studies

In order to achieve overexpression of *trem1* in Raw264.7 cells, these cells were transfected with the pCMV-*trem1* construct or with an empty pCMV vector as a control. To inhibit trem1 expression in Raw264.7 cells, aliquots were incubated with *trem1* siRNA oligo duplex (OriGene, Rockville, MD; catalog #SR406370) or with "trilencer-27 universal scrambled negative control" as a control (OriGene, Rockville, MD; catalog#SR30004).

siRNA-duplexes were obtained as annealed oligos and transfected at a final concentration of 200 nM. RAW264.7 cells were transfected using the lipofectamine 3000 transfection kit (Invitrogen, Waltham, MA; catalog# L3000008) according to the manufacturer's protocol, and used at 24 h post-transfection, which was the predetermined optimal timepoint.

Stored RBC supernatant

Stored RBCs were prepared as in the experimental design above using FVB-donor mice, and supernatant was prepared from stored RBCs by centrifugation at 200 x g for 10 minutes. Fresh supernatants were used as negative controls.

Reverse Transcriptase-Quantitative PCR

Primers (Table 1) were designed using Beacon Design software (Bio-Rad, Hercules, CA). We used a standard reverse transcriptase reaction and a SYBR green-based method to measure mRNA expression[30]. Data were normalized against 18s ribosomal RNA.

Western blotting

TLR4 and trem1 expression in the macrophage cell line was measured using our previously described protocol [31, 32] with anti-TLR4 rabbit polyclonal antibody (Proteintech, Rosemont, IL; catalog# 19811–1-AP1; 1:1000 dilution), anti-trem1-rabbit polyclonal antibody (Abcam, Waltham, MA; catalog# AB214202; 1:800 dilution), goat anti-rabbit IgG HRP conjugate (Bio-Rad, Boulder, CO; catalog# 1721019; 1:5000 dilution), and goat anti-mouse IgG HRP conjugate (Bio-Rad, Boulder, CO; catalog# 17211011; 1:5000 dilution) were used for Western blotting experiments. Data were normalized against β-actin (Santacruz, Dallas, TX; catalog# Sc-8423; 1:500 dilution). Membranes were developed using ECL prime Western blotting detection reagents (Amersham, Cytiva, Little Chalfont, UK; catalog# RPN2332).

Statistical methods.

Statistical analyses were performed using GraphPad Prism software, version 10.0.2 (GraphPad, La Jolla, CA, USA). All tests were two-sided. Differences were considered significant at $P < 0.05$.

Results

RBCTs induce an acute inflammatory response in the blood of anemic neonates.

To explore the mechanism(s) involved in anemia-RBC transfusion-associated SIRS, we used a murine model described previously[6] that included four test groups: (a) naïve control; (b) transfusion control; (c) anemic control; and (d) anemic-transfused mice. Endotoxin-free and free-heme enriched (Suppl Fig 1a–b) stored RBCTs led to significant increases in plasma levels of proinflammatory cytokines (IFN-γ, IL-1β, TNF-α, IL-6, MIP1α, MIP1β, MIP2, LIX) within 2 hours post-transfusion (Fig. 1a–h) in control pups with normal hematocrit ("transfusion controls"), as well as in anemic pups (anemic-transfused). In comparison, these cytokine levels were significantly higher in anemic-transfused pups and remained elevated at 6 hours; however, in the transfusion controls this response predominantly

returned to baseline after 2 hours. We ignored the inflammatory response after 6 hours post-transfusion because intestinal inflammation (i.e., RBC-transfusion-associated necrotizing enterocolitis [TANEC]) was observed after that time as evidenced by the release of intestinal fatty acid binding protein (iFABP) (Fig. 1i). Acute clearance of effete stored RBCs delivers large amounts of iron to the mononuclear phagocyte system[23]. To this end, we measured plasma NTBI levels; although NTBI levels increased post-transfusion, no differences were found when comparing transfusion control and anemic-transfused pups (Fig. 1j), indicating that the characteristics of the acute inflammatory response in anemic-transfused mice were independent of NTBI production due to clearance of transfused stored RBCs. Lacking convincing support for iron-induced SIRS following stored RBCT in anemic-transfused mice, we explored whether anemia-associated "leaky gut" leads to circulating endotoxin levels, which may sensitize circulating monocytes. To investigate whether increasing pre-transfusion anemia may increase the severity of RBCT-associated acute inflammatory responses, we measured hematocrit, intestinal permeability to FITC-dextran, and plasma endotoxin in every phlebotomy. Intestinal permeability (Fig. 1k) and plasma endotoxin levels were simultaneously elevated from day 5 onwards and directly correlated with decreasing hematocrit, consistent with our previous findings[5] that severe anemia alters gut barrier function and increases plasma endotoxin levels due to bacterial translocation across the anemic intestine.

Severe anemia increases circulating monocyte levels and induces synthesis and intracellular retention of proinflammatory cytokines.

We recently described[6] that macrophage precursors like monocytes infiltrated into the anemic hypoxic intestine, and were then activated by RBCTs *via* a TLR-4-mediated mechanism, thereby causing bowel injury over the ensuing 12–24 hours. Based on this observation, and by the acute rise (i.e., within 2 hours) in circulating inflammatory cytokines in anemic-transfused pups, we postulated that circulating monocytes might be primed by endotoxin during anemia and induce a proinflammatory response by synthesizing and retaining cytokines intracellularly that can then be released acutely upon stimulation by stored RBCTs. Consistent with this hypothesis, flow cytometry analysis of blood samples from control anemic mouse pups showed that synthesis of inflammatory cytokines was induced in circulating monocytes. By flow cytometry, we also found that $CD11b^+$ myeloid cells were slightly reduced in anemic blood; however, $CD11b^+Ly6C^+$ monocytes were significantly increased with no or minimal changes in numbers of $CD11b^+F4/80^+$ macrophages (Fig. 2a–b). Furthermore, using flow cytometry-based intracellular staining of $Ly6C^+$ monocytes for major proinflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-1β,) in the blood of control and anemic mice(Fig. 2c–f), we found that inflammatory cytokines were pre-synthesized within circulating monocytes as the levels of each cytokine in $Ly6C^+$ cells were significantly higher in anemic mice, as compared to controls. Indeed, the mRNA levels of major proinflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-1β) were significantly increased in $Ly6C^+$ monocytes derived from anemic blood than controls (Supp Fig 2).

RBCTs promote a TLR4-independent SIRS in anemic murine neonates.

RBCTs in anemic murine pups mediate gut mucosal injury through TLR4 activation and the absence of TLR4 on macrophages is protective[6]. Therefore, to investigate whether

TLR4 activation was involved in stored RBCT-associated acute inflammatory responses, $TLR4^{-/-}$ anemic pups and $TLR4^{-/-}$ control pups received RBCTs, and plasma levels of proinflammatory cytokines were measured over the ensuing 2–6 hours. Overall, although plasma levels of proinflammatory cytokines (IFN-γ, IL-1β, TNF-α, IL-6,) were reduced as compared to their anemic-transfused WT-littermates (not shown) they remained more elevated in anemic-transfused $TLR4^{-/-}$ murine pups as compared to $TLR4^{-/-}$ transfusion control pups (Supp Fig 3a–d), indicating that this acute proinflammatory response does not depend on TLR4- dependent signaling.

Severe anemia induces *trem1* on circulating monocytes and triggers the synthesis of key inflammatory genes.

Lacking support for TLR4-associated SIRS during anemia and/or with associated RBCT, we focused on endotoxin receptors on circulating monocytes in anemia. To identify endotoxin receptors that are modulated in circulating monocytes during anemia, we isolated $LY6C^{+}$ monocytes using magnetic microbeads and examined mRNA expression by qRT-PCR analysis of multiple endotoxin receptors, including LBP (lipopolysaccharide binding protein), MD2 (myeloid differentiation factor-2), CD14 (cluster of differentiation 14), RAGE (receptor for advanced glycation endproducts), HMGB1 (high mobility group box 1), trem1 (triggering receptor expressed on myeloid cells 1), TLR2 (toll-like receptor 2), and TLR4. Interestingly, the circulating monocytes in anemic mice expressed significantly elevated mRNA encoding the LBP, MD2, trem1, and TLR2 receptors, as compared to the control. However, minimal expression of mRNA encoding CD14, and none encoding RAGE, HMGB1 and TLR4, were noted. The effect on anemic-mediated *trem1* mRNA expression was more significant than the other markers, with ~2.8-fold increases as compared to controls (Fig. 3a). Consistent with the qRT-PCR results, flow cytometry also confirmed increased expression of *trem1* on the surface of $Ly6C^{+}$ monocytes obtained from anemic mice, as compared to few monocytes expressing *trem1* in control mice[8% ± 0.7 in control *vs* 74% ± 6.8 in anemia; $p<0.01$] (Fig. 3b). Trem1 is one of the most important LPS receptors associated with inflammation[33–35] and, considering its potential importance, we focused on its expression and regulation during anemia-RBC transfusion-associated SIRS.

Our next goal was to identify whether *trem1* mRNA expression was induced by endotoxin-related low-grade inflammation using the RAW264.7 murine macrophage cell line. To this end, cells were treated with LPS at different concentrations (0.1 μg/ml-100 μg/ml) for varying time intervals (4–24 hours). Using qRT-PCR (Fig. 3c), *trem1* mRNA expression was induced using 0.5 μg/ml of LPS at 4 hours. However, there was minimal *trem1* expression in 24 hours with the same dose of LPS treatment. We further confirmed the trem1 protein expression by Western blotting and found that 0.5 μg/ml of LPS significantly induced trem1 protein expression in 4 hours.

In contrast, LPS did not induce changes in mRNA or protein expression of TLR4 in these cells within 4 hours. However, TLR4 expression was significantly higher at 24 hours (Supp Fig.4a), indicating that the LPS-induced inflammatory response affecting TLR4 expression might have occurred at delayed time points, but *trem1* expression was initiated earlier. To support these findings, mRNA expression encoding pro-inflammatory cytokines (TNF-α,

IFN-γ, IL-1β, IL-6) also significantly increased by 4 hours (Fig. 3e–h) indicating that LPS induction within 4 hours can facilitate *trem1* expression by increasing proinflammatory cytokine synthesis through TLR4-independent signaling using NF-kB1 and NF-kB2 (Suppl Fig. 4b, c). Seeking additional evidence that *trem1* expression is needed for proinflammatory cytokine synthesis, we next overexpressed the *trem1*-containing plasmid in RAW264.7 cells demonstrating overexpression of *trem1* mRNA and its encoded protein (Fig. 3i), along with increased expression of proinflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6) (Fig. 3j, k, l, n). In addition, there was no change in expression of TLR4 mRNA (Fig. 3m), whereas the expression levels of mRNAs encoding NF-kB1 and NF-kB2 were significantly increased (Fig. 3o, p).

Acute release of preformed proinflammatory cytokines by monocytes is induced by stored RBC supernatant.

We next sought the molecular mechanism(s) by which stored RBC product(s) caused an acute release of proinflammatory cytokines and SIRS. To this end, we measured the potential activity of stored RBCs supernatant in inducing cytokine release *in vitro* by sensitizing RAW264.7 cells with 0.5 μg/mL of LPS for 4 hours, then exposing them to 50 μL of stored undiluted RBCs supernatant. qRT-PCR and Western blot analysis (Fig. 4a) confirmed that LPS sensitization led to increased expression of *trem1* mRNA and trem1 protein, respectively. Interestingly, exposure to stored RBC supernatant alone increased *trem1* expression and this was expressed at an even higher level in LPS-sensitized RAW246.7 cells. We also performed qRT-PCR for proinflammatory cytokines (i.e., TLR4, NFκB-1, NFκB-2 IFN-γ, IL-1β, TNF-α and IL-6) (Suppl Fig. 5a–g) in cells treated for 4 hours with LPS and stored RBCs supernatant for 2 hours. Surprisingly, the proinflammatory cytokines were significantly higher when treated with both 4 hours of LPS and 2 hours of stored RBCs supernatant, whereas TLR4 expression was also higher with NF-kB1 and NF-kB2, showing that this combined inflammatory response triggered by stored RBC supernatant is mediated through TLR4 signaling.

To ascertain whether *trem1* plays a causative role in response to stored RBC supernatant, we silenced *trem1* expression in RAW264.7 cells by transfection with *trem1*-siRNA, or with scrambled siRNA as control; the cells were then treated with 0.5 μg/mL of LPS for 4 hours followed by 50 μL of stored RBC supernatant (50 μl for 1×10^{-6} cells) for 2 hours. Decreased *trem1* transcription/translation in these cells confirmed the efficiency of transfection, and significantly decreased *trem1* mRNA and protein expression in RAW264.7 cells, even when treated with LPS and stored RBC supernatant, was confirmed by qRT-PCR and Western blotting, respectively (Fig. 4b). Interestingly, *trem1*-siRNA transfected cells showed reduced expression of IFN-γ, IL-1β, TNF-α, IL-6, NFκB-1, and NFκB-2 but not of TLR4 (Suppl Fig. 6a–g), indicating that *trem1* expression is required for the synthesis of inflammatory cytokines by NF-kB signaling. We studied the effect of *trem1* on acute cytokine release into the extracellular media. Because cytokines are stored in cytoplasmic secretory granules for release induced by cellular activation[36], we tested whether silencing *trem1* expression inhibited cytokine release into the conditioned medium upon treatment with LPS and stored RBC supernatant. As shown in Fig. 4c, the acute release of proinflammatory cytokines (IFN-γ, IL-1β, TNF-α and IL-6) by *trem1*-siRNA transfected cells was significantly suppressed,

as compared to scrambled-siRNA transfected cells, following treatment with LPS and stored RBC supernatant. Taken together, these results demonstrate that *trem1* silencing mitigates rapid, marked reduction of both endotoxin-induced synthesis and intracellular storage of proinflammatory cytokines, as well as the RBC supernatant-induced release of these cytokines.

Myeloid-specific *trem1* inhibition reduces anemic-transfusion-associated SIRS.

To understand the role of *trem1* in anemia-RBC transfusion-associated SIRS, we used transgenic mice containing *trem1*$^{flox/flox}$ carrying a loxP-flanked transcriptional Stop element and then crossed these with Lyz2-cre mice to delete the floxed Stop element and thereby delete *trem1* on their littermates' monocytes and/or macrophages (Fig. 5a). In these mouse pups, flow cytometry confirmed that *trem1* was selectively absent in anemic monocytes (Fig. 5b). The *trem1*$^{-/-}$ mice were divided into four groups, as above, and circulating proinflammatory cytokine levels were measured 2–6 hours after administering stored RBCTs. Proinflammatory cytokine levels (Fig. 5c–j; IFN-γ, IL-1β, TNF-α, IL-6, MIP1α, MIP1β, MIP2, LIX) were significantly lower compared to WT-anemic transfused littermates, even after 4 hours, and further lowered as compared to the baseline in *trem1*$^{-/-}$ anemic-transfused mice. In addition, the induced fit docking module of SCHRODINGER (Supp Fig 7) showed that heme may form pi-pi stacking interaction with Tyr111 and Phe85, cation-pi interaction with Arg57, and salt-bridge interaction with Arg57 of trem1. These findings were of interest because this study strongly suggests that silencing or inhibiting *trem1* expression may provide a potential therapeutic approach to ameliorate both transfusion-associated SIRS and the anemia-related early inflammatory response.

Discussion

We recently developed a murine model of RBCT-associated NEC-like injury, describing that severe anemia in neonates leads to the development of low-grade inflammation in the intestine with prominent macrophage infiltration and that RBCTs can activate these cells to cause NEC-like intestinal injury at 12–24 hours post-transfusion. We now present a detailed investigation into the mechanism(s) by which endotoxin sensitizes circulating monocytes in these anemic murine neonates, enabling stored RBCTs then to immediately induce SIRS via *trem1* signaling. These RBCTs trigger an acute cytokine storm by 2 hours post-transfusion in anemic mice, but not in controls, which exhibit a steady increase up to 6 hours. Anemic murine neonates are uniquely predisposed to SIRS because disrupted epithelial barriers allow bacterial translocation into the intestinal *lamina propria* and the associated bloodstream, thereby triggering a disproportionately severe and dysregulated response in which monocytes synthesize and store cytokines in secretory granules, which then are released following a stored RBCT. This is particularly relevant because bacterial translocation frequently occurs in critically ill patients[37] of all ages, suggesting that our model of neonatal anemic-transfusion-associated SIRS may aid in developing novel anti-inflammatory therapies to prevent or ameliorate this complication of RBCT. A clinical study produced evidence for sex-specific differences in inflammatory cytokine responses to RBC transfusion in preterm infants in the neonatal period[38]. However, we didn't observe a

significant change in major inflammatory cytokines between male and female anemic mouse pups after RBC transfusion.

We detected increased *trem1* expression on circulating monocytes in anemia due to endotoxin exposure and observed that inflammatory cytokine synthesis depended on *trem1*, but not TLR4. In endotoxemia, the resulting inflammatory process is initially beneficial to the host as a means of protecting against invading microorganisms; however, RBCTs induced exocytosis of proinflammatory cytokines which was detrimental to the host. Although the classic endotoxin signaling pathway involves LBP, CD14, MD-2, and TLR4, alternative mediators, like *trem1*, can also be involved. *Trem1* is constitutively expressed on most monocytes, macrophages and neutrophils[39], is a critical amplifier in the innate immune response[40–42], and shares the downstream NF-kB signaling pathway with TLR4[43–46]. In our study, *trem1* expression correlated with the synthesis of inflammatory cytokines, possibly mediated through NF-kB signaling, without involving TLR4, because $TLR4^{-/-}$ mice displayed a similar pattern of SIRS during anemia-RBCT.

Packed RBCs undergo progressive biochemical and morphological changes during refrigerated storage that result in oxidative stress, the generation of reactive oxygen species, damage to proteins and lipids, and the release of cytosolic constituents (e.g., heme, hemoglobin) into stored RBC supernatant[23, 47–51]. Although free heme can directly activate TLR4-mediated signaling[52–55], LPS[56] and heme likely use different binding sites on TLR4. In support, we detected enhanced expression of *trem1* followed by increased synthesis of inflammatory cytokines in cells treated with LPS and their acute release induced by stored RBCs-supernatant treatment might be triggered by free hemoglobin binding with *trem1*. Because of the lack of convincing data that SIRS-associated inflammatory response is TLR4-independent, and by molecular docking, our findings strongly support the hypothesis that *trem1* could be the receptor for free hemoglobin, and further study is needed to investigate this potential mechanism in the release of cytokines from monocytes Consistent with this, *trem1*-silenced macrophages fail to synthesize and release inflammatory cytokines in response to LPS and stored RBCs-supernatant. While *trem1* interacts with the TLR4 receptor complex or is a component of this complex, simultaneous stimulation of TLR4 and *trem1* activating receptors on monocytes and neutrophils amplifies the inflammatory signal and increases in production of multiple proinflammatory cytokines. Our findings are consistent with the findings that t*rem1* amplified not only the TLR-dependent signals but also the activation of NLR-dependent pathways that lead to the induction of the production of the proinflammatory cytokines[57], but these findings need further examination.

We found that *trem1* silencing *in vitro* prevented LPS- and stored RBCs-supernatant-induced production and secretion of proinflammatory cytokines; similarly, myeloid-specific deletion of *trem1* in mice analogously reduced acute inflammation *in vivo* following neonatal RBCTs. Silencing and/or blockade of *trem1* on myeloid cells also decreases the detrimental effects of inflammatory responses in other settings, including in sepsis[58–61], cancer inflammation[62–65], colitis[39, 66, 67], myocardial infarction[68], stroke[69], and atherosclerosis[70]. Given that there are no effective, currently available treatments for transfusion-associated SIRS, this model may provide a viable platform for evaluating new

therapeutic approaches. For example, a *trem1*-inhibitor peptide, *Nangibotide*, is currently being evaluated (www.clinicaltrials.gov, NCT03158948) for septic shock and myocardial infarction. Overall, the current study provides new insights into inflammatory signaling through *trem1*, suggesting unique effects on neonatal health.

In conclusion, anemia-associated low-grade inflammation sensitized circulating monocytes to produce and store proinflammatory cytokines, which was mediated by *trem1*. Stored RBCTs then stimulated the release of these preformed cytokines, leading to SIRS. Inhibiting *trem1* function in myeloid cells, including monocytes, alleviated this anemia- and RBCT-associated inflammatory response in murine neonates. Further studies to delineate the specific molecular mechanisms involved in regulating *trem1* function will not only lead to greater understanding but will also suggest novel therapeutic interventions to increase the safety and efficacy of RBCTs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Arthur CM, Nalbant D, Feldman HA, Saeedi BJ, Matthews J, Robinson BS, et al. Anemia induces gut inflammation and injury in an animal model of preterm infants. Transfusion 2019; 59:1233–1245. [PubMed: 30897226]
2. Cibulskis CC, Maheshwari A, Rao R, Mathur AM. Anemia of prematurity: how low is too low? J Perinatol 2021; 41:1244–1257. [PubMed: 33664467]
3. Higgins RD, Patel RM, Josephson CD. Preoperative Anemia and Neonates. JAMA Pediatr 2016; 170:835–6. [PubMed: 27428663]
4. Maheshwari A, Patel RM, Christensen RD. Anemia, red blood cell transfusions, and necrotizing enterocolitis. Semin Pediatr Surg 2018; 27:47–51. [PubMed: 29275817]
5. MohanKumar K, Namachivayam K, Sivakumar N, Alves NG, Sidhaye V, Das JK, et al. Severe neonatal anemia increases intestinal permeability by disrupting epithelial adherens junctions. Am J Physiol Gastrointest Liver Physiol 2020; 318:G705–G716. [PubMed: 32090604]
6. MohanKumar K, Namachivayam K, Song T, Jake Cha B, Slate A, Hendrickson JE, et al. A murine neonatal model of necrotizing enterocolitis caused by anemia and red blood cell transfusions. Nat Commun 2019; 10:3494. [PubMed: 31375667]
7. Saroha V, Josephson CD, Patel RM. Epidemiology of Necrotizing Enterocolitis: New Considerations Regarding the Influence of Red Blood Cell Transfusions and Anemia. Clin Perinatol 2019; 46:101–117. [PubMed: 30771812]

8. Whitehead HV, Vesoulis ZA, Maheshwari A, Rambhia A, Mathur AM. Progressive anemia of prematurity is associated with a critical increase in cerebral oxygen extraction. Early Hum Dev 2020; 140:104891. [PubMed: 31669878]
9. Whitehead HV, Vesoulis ZA, Maheshwari A, Rao R, Mathur AM. Anemia of prematurity and cerebral near-infrared spectroscopy: should transfusion thresholds in preterm infants be revised? J Perinatol 2018; 38:1022–1029. [PubMed: 29740185]
10. Rosebraugh MR, Widness JA, Nalbant D, Veng-Pedersen P. A mathematical modeling approach to quantify the role of phlebotomy losses and need for transfusions in neonatal anemia. Transfusion 2013; 53:1353–60. [PubMed: 23033916]
11. Whyte RK, Jefferies AL, Canadian Paediatric Society F, Newborn C. Red blood cell transfusion in newborn infants. Paediatr Child Health 2014; 19:213–22. [PubMed: 24855419]
12. Baxi AC, Josephson CD, Iannucci GJ, Mahle WT. Necrotizing enterocolitis in infants with congenital heart disease: the role of red blood cell transfusions. Pediatr Cardiol 2014; 35:1024–9. [PubMed: 24626816]
13. Elabiad MT, Harsono M, Talati AJ, Dhanireddy R. Effect of birth weight on the association between necrotising enterocolitis and red blood cell transfusions in <=1500 g infants. BMJ Open 2013; 3:e003823.
14. Josephson CD, Wesolowski A, Bao G, Sola-Visner MC, Dudell G, Castillejo MI, et al. Do red cell transfusions increase the risk of necrotizing enterocolitis in premature infants? J Pediatr 2010; 157:972–978 e1–3. [PubMed: 20650470]
15. Chirico G, Beccagutti F, Sorlini A, Motta M, Perrone B. Red blood cell transfusion in preterm infants: restrictive versus liberal policy. J Matern Fetal Neonatal Med 2011; 24 Suppl 1:20–2. [PubMed: 21942584]
16. Howarth C, Banerjee J, Aladangady N. Red Blood Cell Transfusion in Preterm Infants: Current Evidence and Controversies. Neonatology 2018; 114:7–16. [PubMed: 29550819]
17. Villeneuve A, Arsenault V, Lacroix J, Tucci M. Neonatal red blood cell transfusion. Vox Sang 2021; 116:366–378. [PubMed: 33245826]
18. Abdelghaffar S, Mansi Y, Ibrahim R, Mohamed D. Red blood transfusion in preterm infants: changes in glucose, electrolytes and acid base balance. Asian J Transfus Sci 2012; 6:36–41. [PubMed: 22623841]
19. Aucott SW, Maheshwari A. To transfuse or not transfuse a premature infant: the new complex question. J Perinatol 2019; 39:351–353. [PubMed: 30651578]
20. Aubron C, Nichol A, Cooper DJ, Bellomo R. Age of red blood cells and transfusion in critically ill patients. Ann Intensive Care 2013; 3:2. [PubMed: 23316800]
21. Escobar GA, Cheng AM, Moore EE, Johnson JL, Tannahill C, Baker HV, et al. Stored packed red blood cell transfusion up-regulates inflammatory gene expression in circulating leukocytes. Ann Surg 2007; 246:129–34. [PubMed: 17592301]
22. Gauvin F, Spinella PC, Lacroix J, Choker G, Ducruet T, Karam O, et al. Association between length of storage of transfused red blood cells and multiple organ dysfunction syndrome in pediatric intensive care patients. Transfusion 2010; 50:1902–13. [PubMed: 20456697]
23. Hod EA, Zhang N, Sokol SA, Wojczyk BS, Francis RO, Ansaldi D, et al. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. Blood 2010; 115:4284–92. [PubMed: 20299509]
24. Friedlander MH, Simon R, Machiedo GW. The relationship of packed cell transfusion to red blood cell deformability in systemic inflammatory response syndrome patients. Shock 1998; 9:84–8. [PubMed: 9488251]
25. Kasivajjula H, Maheshwari A. Pathophysiology and current management of necrotizing enterocolitis. Indian J Pediatr 2014; 81:489–97. [PubMed: 24652270]
26. Pammi M, De Plaen IG, Maheshwari A. Recent Advances in Necrotizing Enterocolitis Research: Strategies for Implementation in Clinical Practice. Clin Perinatol 2020; 47:383–397. [PubMed: 32439118]
27. Chung Y, Desiraju S, Namachivayam K, Guzman P, He L, MohanKumar K. Hematological changes in neonatal mice with phlebotomy-induced anemia. Pediatr Res 2022; 92:1575–1579. [PubMed: 35322186]

28. Ramatchandirin B, Balamurugan MA, Desiraju S, Chung Y, MohanKumar K. A Detailed Protocol for the Induction of Anemia and RBC Transfusion-associated Necrotizing Enterocolitis in Neonatal Mice. Bio Protoc 2024; 14:e4993.
29. Evans RW, Rafique R, Zarea A, Rapisarda C, Cammack R, Evans PJ, et al. Nature of non-transferrin-bound iron: studies on iron citrate complexes and thalassemic sera. J Biol Inorg Chem 2008; 13:57–74. [PubMed: 17906879]
30. MohanKumar K, Kaza N, Jagadeeswaran R, Garzon SA, Bansal A, Kurundkar AR, et al. Gut mucosal injury in neonates is marked by macrophage infiltration in contrast to pleomorphic infiltrates in adult: evidence from an animal model. Am J Physiol Gastrointest Liver Physiol 2012; 303:G93–102. [PubMed: 22538401]
31. MohanKumar K, Namachivayam K, Chapalamadugu KC, Garzon SA, Premkumar MH, Tipparaju SM, et al. Smad7 interrupts TGF-beta signaling in intestinal macrophages and promotes inflammatory activation of these cells during necrotizing enterocolitis. Pediatr Res 2016; 79:951–61. [PubMed: 26859364]
32. Namachivayam K, Blanco CL, MohanKumar K, Jagadeeswaran R, Vasquez M, McGill-Vargas L, et al. Smad7 inhibits autocrine expression of TGF-beta2 in intestinal epithelial cells in baboon necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 2013; 304:G167–80. [PubMed: 23154975]
33. Shen L, Zhou Y, Wu X, Sun Y, Xiao T, Gao Y, et al. TREM1 Blockade Ameliorates Lipopolysaccharide-Induced Acute Intestinal Dysfunction through Inhibiting Intestinal Apoptosis and Inflammation Response. Biomed Res Int 2021; 2021:6635452. [PubMed: 33954188]
34. Suh HC, Benoukraf T, Shyamsunder P, Yin T, Cao Q, Said J, et al. LPS independent activation of the pro-inflammatory receptor Trem1 by C/EBPepsilon in granulocytes. Sci Rep 2017; 7:46440. [PubMed: 28440307]
35. Zanzinger K, Schellack C, Nausch N, Cerwenka A. Regulation of triggering receptor expressed on myeloid cells 1 expression on mouse inflammatory monocytes. Immunology 2009; 128:185–95. [PubMed: 19740375]
36. Lacy P, Stow JL. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. Blood 2011; 118:9–18. [PubMed: 21562044]
37. Otani S, Coopersmith CM. Gut integrity in critical illness. J Intensive Care 2019; 7:17. [PubMed: 30923621]
38. Benavides A, Bell EF, Georgieff MK, Josephson CD, Stowell SR, Feldman HA, et al. Sex-specific cytokine responses and neurocognitive outcome after blood transfusions in preterm infants. Pediatr Res 2022; 91:947–954. [PubMed: 33911194]
39. Schenk M, Bouchon A, Seibold F, Mueller C. TREM-1--expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. J Clin Invest 2007; 117:3097–106. [PubMed: 17853946]
40. Dower K, Ellis DK, Saraf K, Jelinsky SA, Lin LL. Innate immune responses to TREM-1 activation: overlap, divergence, and positive and negative cross-talk with bacterial lipopolysaccharide. J Immunol 2008; 180:3520–34. [PubMed: 18292579]
41. Roe K, Gibot S, Verma S. Triggering receptor expressed on myeloid cells-1 (TREM-1): a new player in antiviral immunity? Front Microbiol 2014; 5:627. [PubMed: 25505454]
42. Arts RJ, Joosten LA, Dinarello CA, Kullberg BJ, van der Meer JW, Netea MG. TREM-1 interaction with the LPS/TLR4 receptor complex. Eur Cytokine Netw 2011; 22:11–4. [PubMed: 21393102]
43. Carrasco K, Boufenzer A, Jolly L, Le Cordier H, Wang G, Heck AJ, et al. TREM-1 multimerization is essential for its activation on monocytes and neutrophils. Cell Mol Immunol 2019; 16:460–472. [PubMed: 29568119]
44. Zhang M, Yin C, Chen Y, Wang J, Jiang J. Upstream stimulatory factor 2 (USF2) induced upregulation of triggering receptor expressed on myeloid cells 1 (TREM1) promotes endometritis by regulating toll-like receptor (TLR) 2/4-nuclear factor-kappaB (NF-kappaB) signaling pathway. Bioengineered 2022; 13:3609–3619. [PubMed: 35100093]

45. Yan H, Li H, Zhu L, Gao J, Li P, Zhang Z. Increased TLR4 and TREM-1 expression on monocytes and neutrophils in preterm birth: further evidence of a proinflammatory state. J Matern Fetal Neonatal Med 2019; 32:2961–2969. [PubMed: 29534643]
46. Zeng H, Ornatowska M, Joo MS, Sadikot RT. TREM-1 expression in macrophages is regulated at transcriptional level by NF-kappaB and PU.1. Eur J Immunol 2007; 37:2300–8. [PubMed: 17634956]
47. Nogueira D, Rocha S, Abreu E, Costa E, Santos-Silva A. Biochemical and cellular changes in leukocyte-depleted red blood cells stored for transfusion. Transfus Med Hemother 2015; 42:46–51. [PubMed: 25960715]
48. Cluitmans JC, Chokkalingam V, Janssen AM, Brock R, Huck WT, Bosman GJ. Alterations in red blood cell deformability during storage: a microfluidic approach. Biomed Res Int 2014; 2014:764268. [PubMed: 25295273]
49. Cluitmans JC, Hardeman MR, Dinkla S, Brock R, Bosman GJ. Red blood cell deformability during storage: towards functional proteomics and metabolomics in the Blood Bank. Blood Transfus 2012; 10 Suppl 2:s12–8. [PubMed: 22890263]
50. Hod EA, Spitalnik SL. Stored red blood cell transfusions: Iron, inflammation, immunity, and infection. Transfus Clin Biol 2012; 19:84–9. [PubMed: 22682673]
51. Sparrow RL. Red blood cell storage and transfusion-related immunomodulation. Blood Transfus 2010; 8 Suppl 3:s26–30. [PubMed: 20606746]
52. Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. Blood 2014; 123:377–90. [PubMed: 24277079]
53. Janciauskiene S, Vijayan V, Immenschuh S. TLR4 Signaling by Heme and the Role of Heme-Binding Blood Proteins. Front Immunol 2020; 11:1964. [PubMed: 32983129]
54. Lin S, Yin Q, Zhong Q, Lv FL, Zhou Y, Li JQ, et al. Heme activates TLR4-mediated inflammatory injury via MyD88/TRIF signaling pathway in intracerebral hemorrhage. J Neuroinflammation 2012; 9:46. [PubMed: 22394415]
55. Wei X, Zhang F, Cheng D, Wang Z, Xing N, Yuan J, et al. Free heme induces neuroinflammation and cognitive impairment by microglial activation via the TLR4/MyD88/NF-kappaB signaling pathway. Cell Commun Signal 2024; 22:16. [PubMed: 38183122]
56. Levy E, Xanthou G, Petrakou E, Zacharioudaki V, Tsatsanis C, Fotopoulos S, et al. Distinct roles of TLR4 and CD14 in LPS-induced inflammatory responses of neonates. Pediatr Res 2009; 66:179–84. [PubMed: 19390489]
57. Netea MG, Azam T, Ferwerda G, Girardin SE, Kim SH, Dinarello CA. Triggering receptor expressed on myeloid cells-1 (TREM-1) amplifies the signals induced by the NACHT-LRR (NLR) pattern recognition receptors. J Leukoc Biol 2006; 80:1454–61. [PubMed: 16940328]
58. Qian L, Weng XW, Chen W, Sun CH, Wu J. TREM-1 as a potential therapeutic target in neonatal sepsis. Int J Clin Exp Med 2014; 7:1650–8. [PubMed: 25126161]
59. Shi X, Zhang Y, Wang H, Zeng S. Effect of Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1) Blockade in Rats with Cecal Ligation and Puncture (CLP)-Induced Sepsis. Med Sci Monit 2017; 23:5049–5055. [PubMed: 29059148]
60. Siskind S, Brenner M, Wang P. TREM-1 Modulation Strategies for Sepsis. Front Immunol 2022; 13:907387. [PubMed: 35784361]
61. Wang F, Liu S, Wu S, Zhu Q, Ou G, Liu C, et al. Blocking TREM-1 signaling prolongs survival of mice with Pseudomonas aeruginosa induced sepsis. Cell Immunol 2012; 272:251–8. [PubMed: 22055202]
62. Bosco MC, Raggi F, Varesio L. Therapeutic Potential of Targeting TREM-1 in Inflammatory Diseases and Cancer. Curr Pharm Des 2016; 22:6209–6233. [PubMed: 27568730]
63. Ho CC, Liao WY, Wang CY, Lu YH, Huang HY, Chen HY, et al. TREM-1 expression in tumor-associated macrophages and clinical outcome in lung cancer. Am J Respir Crit Care Med 2008; 177:763–70. [PubMed: 18096709]
64. Raggi F, Bosco MC. Targeting Mononuclear Phagocyte Receptors in Cancer Immunotherapy: New Perspectives of the Triggering Receptor Expressed on Myeloid Cells (TREM-1). Cancers (Basel) 2020; 12.

65. Shen ZT, Sigalov AB. Novel TREM-1 Inhibitors Attenuate Tumor Growth and Prolong Survival in Experimental Pancreatic Cancer. Mol Pharm 2017; 14:4572–4582. [PubMed: 29095622]

66. Che X, Park KC, Park SJ, Kang YH, Jin HA, Kim JW, et al. Protective effects of guggulsterone against colitis are associated with the suppression of TREM-1 and modulation of macrophages. Am J Physiol Gastrointest Liver Physiol 2018; 315:G128–G139. [PubMed: 29543509]

67. Kokten T, Gibot S, Lepage P, D'Alessio S, Hablot J, Ndiaye NC, et al. TREM-1 Inhibition Restores Impaired Autophagy Activity and Reduces Colitis in Mice. J Crohns Colitis 2018; 12:230–244. [PubMed: 28961797]

68. Boufenzer A, Lemarie J, Simon T, Derive M, Bouazza Y, Tran N, et al. TREM-1 Mediates Inflammatory Injury and Cardiac Remodeling Following Myocardial Infarction. Circ Res 2015; 116:1772–82. [PubMed: 25840803]

69. Xu P, Zhang X, Liu Q, Xie Y, Shi X, Chen J, et al. Microglial TREM-1 receptor mediates neuroinflammatory injury via interaction with SYK in experimental ischemic stroke. Cell Death Dis 2019; 10:555. [PubMed: 31324751]

70. Joffre J, Potteaux S, Zeboudj L, Loyer X, Boufenzer A, Laurans L, et al. Genetic and Pharmacological Inhibition of TREM-1 Limits the Development of Experimental Atherosclerosis. J Am Coll Cardiol 2016; 68:2776–2793. [PubMed: 28007141]

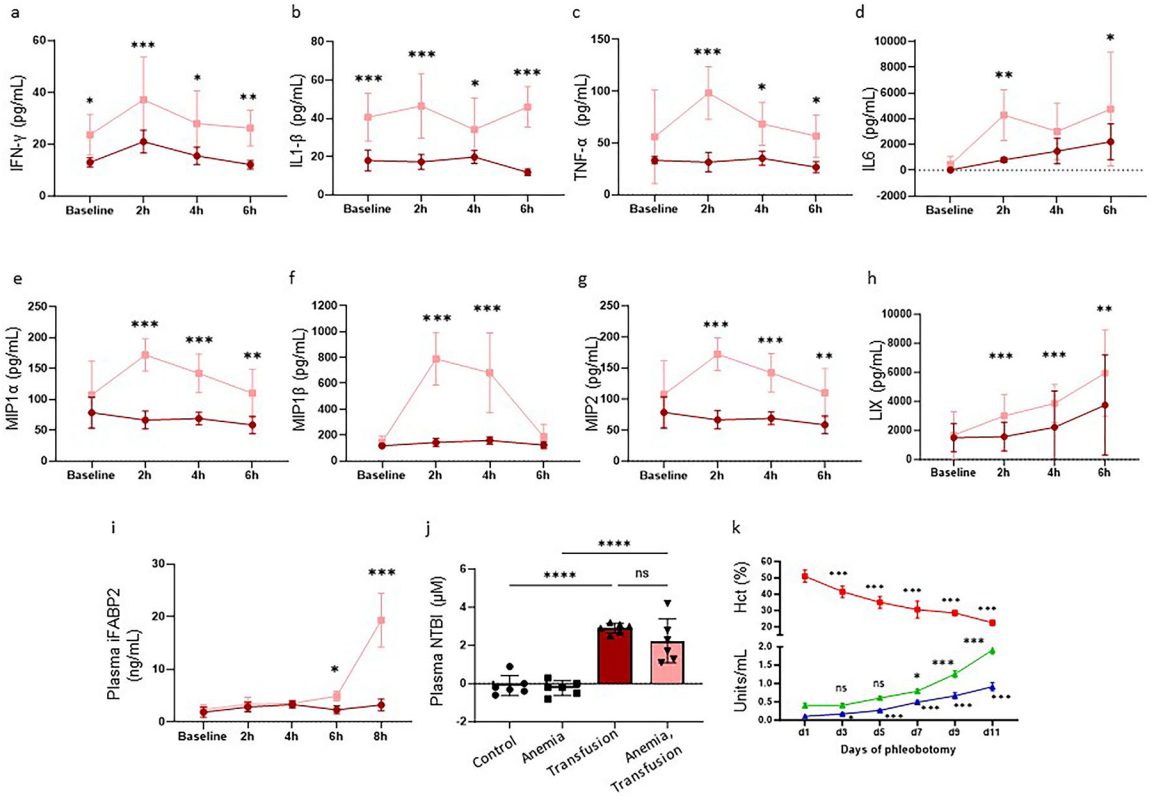


Fig. 1. RBC-transfusion induces an acute inflammatory response in the blood of anemic neonates.

Line diagrams show serial measurements of plasma cytokines (a) IFN-γ, (b) IL-1β, (c) TNF-α, (d) IL6, (e) MIP1α, (f) MIP1β, (g) MIP2 and (h) LIX in transfusion control (maroon lines) and anemic-transfused mice (pink lines). *N*= 8 mice per group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* transfusion control with respective time-points including baseline. (i) The line diagram summarizes plasma intestinal fatty acid-binding protein 2 (iFABP2) concentrations in transfusion control (maroon lines) and anemic-transfused mice (pink lines). *N* =8 mice per group; * $p<0.05$, *** $p<0.001$ *vs* transfusion control with respective time-points including baseline. (j) The scatter bar diagram (means ± SE) shows plasma non-transferrin bound iron (NTBI) in naïve control, anemic control, 2 hours post-transfusion of transfusion control, and anemic-transfused pups on P11. *N*=6 mice per group; ** $p<0.01$; *** $p<0.001$ *vs* Naïve Control. (k) The line (Red) indicates the longitudinal change in hematocrit in the anemia mice during each phlebotomy, which correlates with the increased fluorescence (green line) readings in plasma 4 h after gavage with fluorescein isothiocyanate (FITC)- dextran and increased plasma lipopolysaccharide (LPS) levels during each phlebotomy; N= 8 mice per group. Groups analyzed by Šídák's multiple comparisons test.

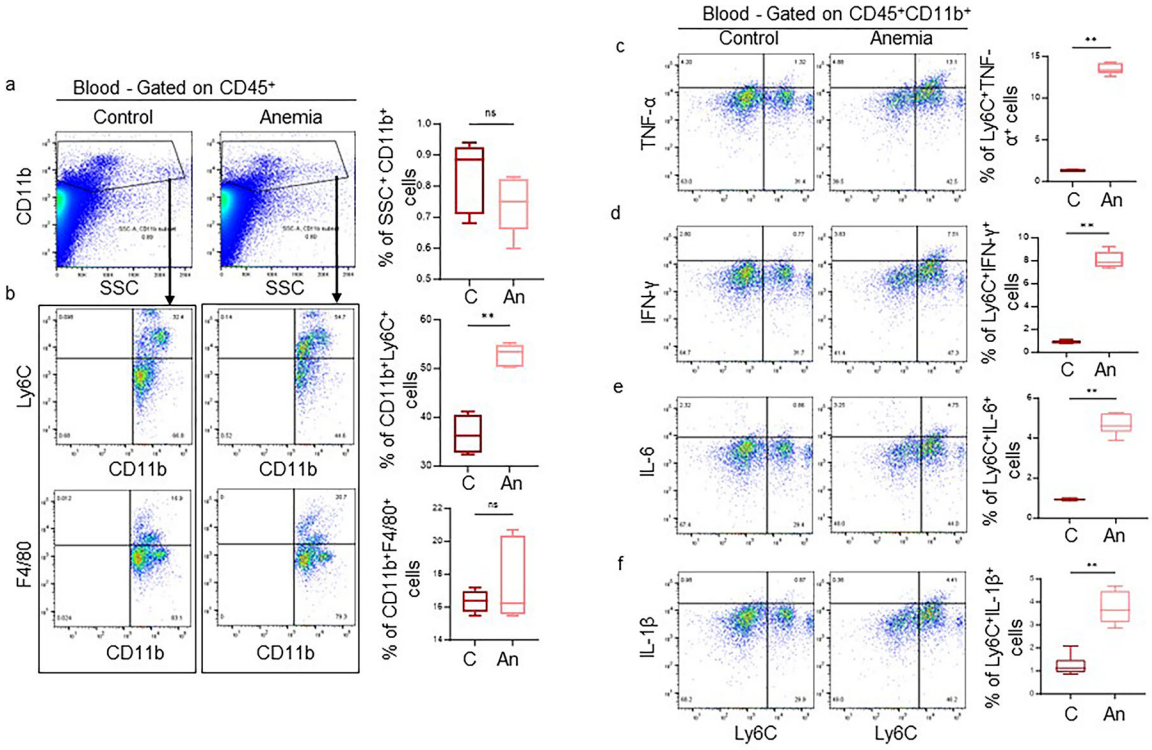
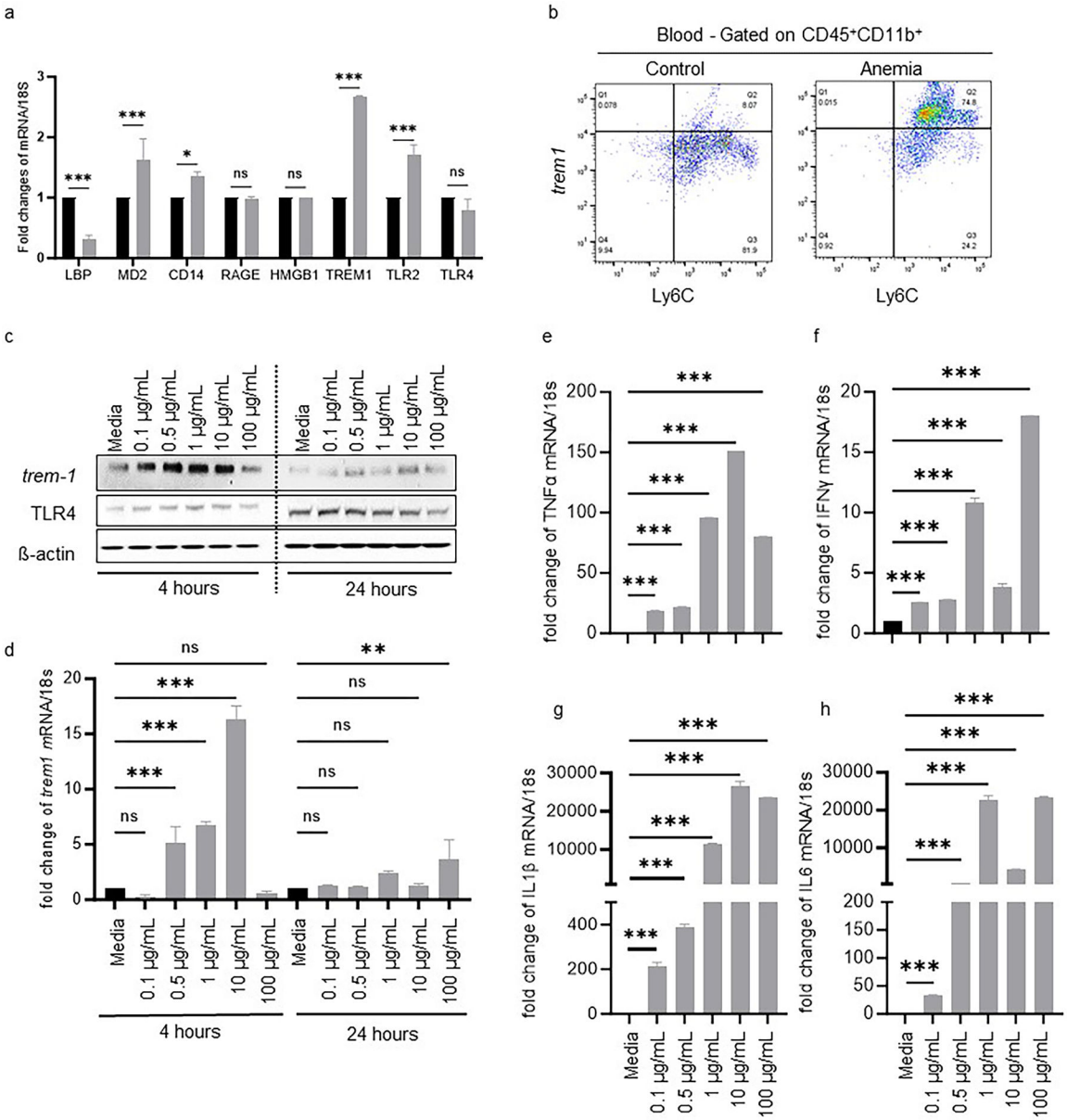


Fig. 2. Severe anemia increases monocyte numbers in blood and induces the preformation of inflammatory cytokines in blood monocytes.

(a) Representative flow cytometry scatter plots from the blood of control and anemic mice. The $CD11b^+$ myeloid cell population from gated $CD45^+$ was slightly decreased during anemia than control and box whisker plots summarize the number of $CD11b^+$ cells in each group. Mann-Whitney U test. (b) Representative flow cytometry scatter plots of the CD11b-positive myeloid cell fraction in control and anemic blood show a $Ly6C^+$ and $F4/80^+$ cell population. Box whisker plots summarize the number of $CD11b^+/Ly6C^+$ and $CD11b^+F4/80^+$ cells. (c-f) Representative flow cytometry scatter plots for TNF-α, IFN-γ, IL6 and IL-1β show intracellular staining with $Ly6C^+$ monocytes from control and anemic blood. All box whisker plots (means ± SE) summarize the proportions of Ly6C monocytes expressing TNF-α, IFN-γ, IL6 and IL-1β. Groups analyzed by Šídák's multiple comparisons test; N=6 mice per group; $^{**}p<0.01$.



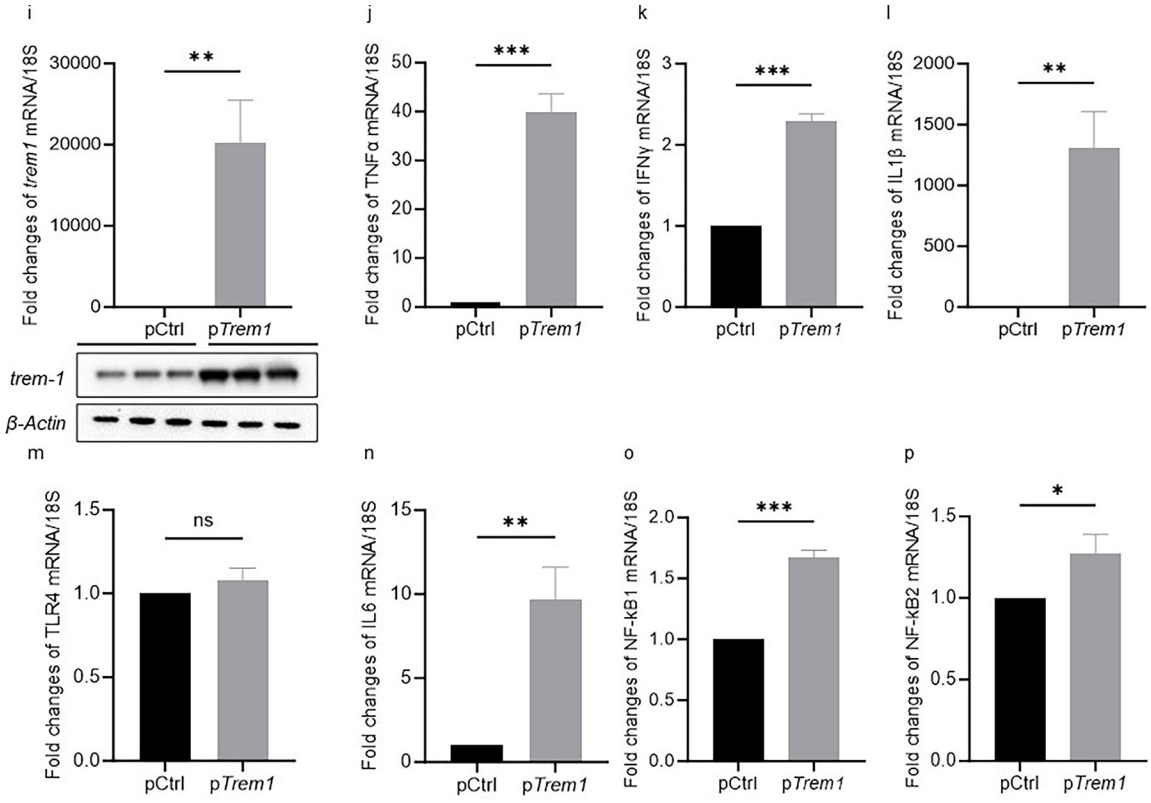


Fig. 3. Severe anemia induces *trem1* on circulating monocytes and triggers the synthesis of key inflammatory genes.

(a) Bar diagram summarizes lipopolysaccharide (LPS)-receptors expression in the $Ly6C^+$ circulating monocytes from control and anemic murine pups measured by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). *N*=6 mice per group; Šídák's multiple comparisons test, *p<0.05, ***p<0.001 *vs* monocytes isolated from the blood of naïve control. (b) Representative flow cytometry scatter plots of the $CD45^+CD11b^+$ myeloid cell population show $Ly6C^+$ monocytes expressing *trem1*$^+$ in the blood of anemic mice than control. (c) Western blots confirm increased *trem1* expression in different concentrations of LPS-treated murine macrophage cells (Raw264.7) within 4 hours compared to 24 hours; however, TLR4 expression was minimal in 4 hours of LPS treatment but increased in 24 hours. Both blots were normalized against β-actin. (d) Bar diagram (mean values ± SEM) represents fold changes of *trem1* mRNA in 4 hours and 24 hours of LPS treatment with murine macrophage cells (Raw264.7). N=3/group; Šídák's multiple comparisons test, **p<0.01, ***p<0.001 *vs* media alone. (e-h) Bar diagram (mean values ± SEM) represents the fold change of mRNAs (e) TNF-α, (f) IFN-γ, (g) IL-1β and (h) IL6 in 4 hours and 24 hours of LPS treatment with murine macrophage cells (Raw264.7). N=3/group; Šídák's multiple comparisons test, ***p<0.001 *vs* media alone. (i-p) Bar diagram (mean values ± SEM) represents the fold change of mRNAs (i) *trem1,* (j) TNF-α, (k) IFN-γ, (l) IL-1β,, (m) TLR4, (n) IL6, (o) NF-kB1 and (p) NF-kB2 in murine macrophage cells (Raw264.7) transfected with plasmid containing *trem1*. (*i)-bottom*- confirms the protein expression of *trem1* in western blot. *N*=3/group; Šídák's multiple comparisons test, *p<0.05, ***p<0.01, ***p*<0.001 *vs* scrambled plasmid transfect.

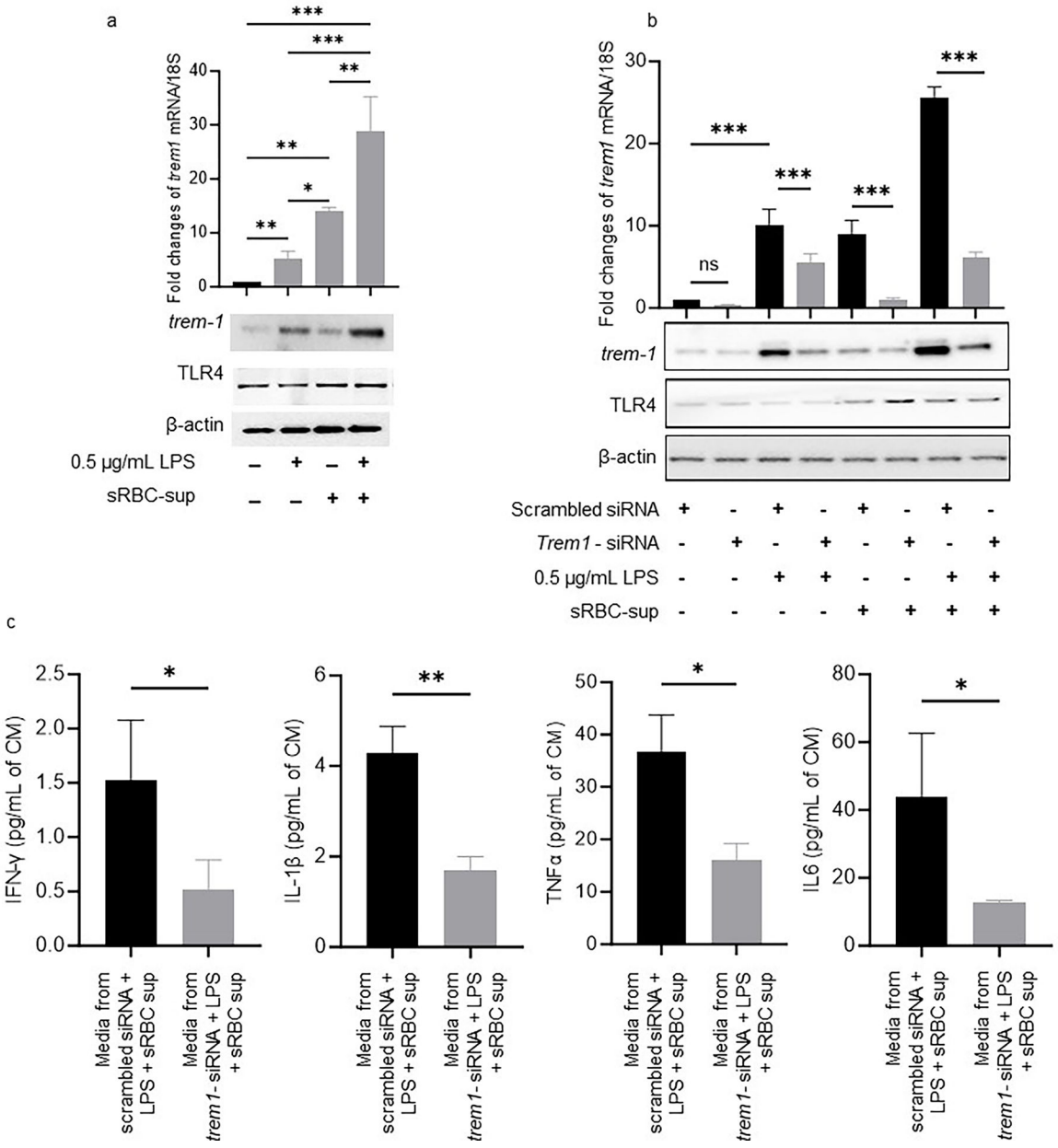


Fig. 4. Acute release of preformed proinflammatory cytokines by monocytes is induced by stored RBC supernatant.

(a-*top*) Bar diagram (mean values ± SEM) represents fold changes of *trem1* mRNA in LPS (0.5 μg/mL of media) and 50 μL of stored RBC supernatant treatment with murine macrophage cells (Raw264.7) for 4 hours. N=3/group; Šídák's multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001 *vs* media alone. (a-*below*) Western blots confirm increased *trem1* expression in LPS (0.5 μg/mL of media) treated with murine macrophage cells (Raw264.7) and further increased during treatment with 50 μL of stored RBC supernatant for 4 hours. Both blots were normalized against β-actin. (b-*top*) Bar diagram (mean values ± SEM) represents fold changes of *trem1* mRNA in murine macrophage cells transfected with siRNA-*trem1* and treated with LPS (0.5 μg/mL of media) and/or with 50 μL of stored

RBC supernatant for 4 hours. N=3/group; Šídák's multiple comparisons test, ***p<0.001 *vs* scrambled siRNA. (b-*below*) Western blots show siRNA-*trem1* treatment diminished the *trem1* expression in murine macrophage cells (Raw264.7) treated with LPS (0.5 μg/mL of media) and/or 50 μL of stored RBC supernatant for 4 hours. Both blots were normalized against β-actin. (c) Bar diagram (mean values ± SEM) represents the levels of cytokines (IFN-γ, IL-1β, TNF-α and IL6) released in the cultured media by murine macrophage cells transfected with either scrambled siRNA or *trem1*-siRNA, then treated 4 hours with *LPS* (0.5 μg/mL of media) and 50 μL of stored RBC supernatant. *N*=3/group; Šídák's multiple comparisons test, *p<0.05, ***p<0.01 vs* scrambled plasmid transfected and treated with LPS, stored RBC supernatant.

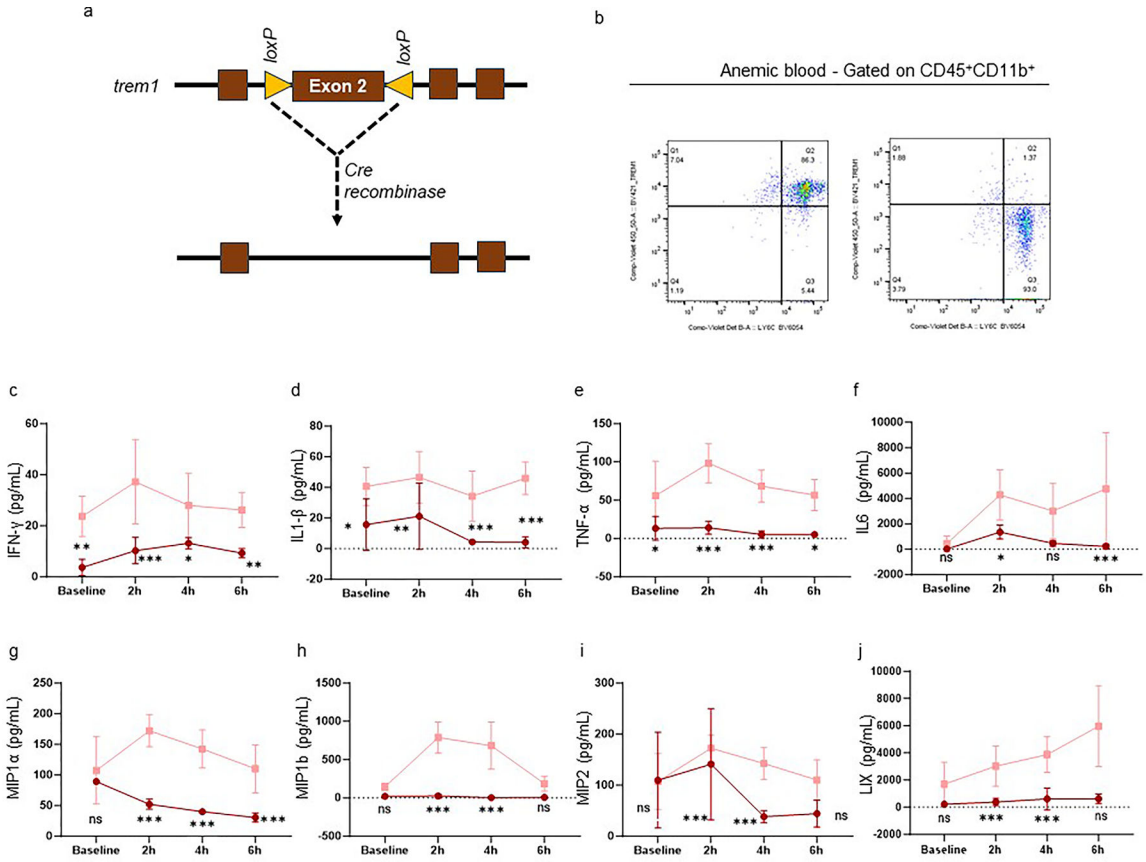


Fig. 5. Myeloid-specific trem1 inhibition reduces anemic-transfusion-associated SIRS.

(a) Schematic of gene targeting strategy. Exon 2 of the *trem1* gene, flanked by LoxP sites, was excised by Lyz2 promoter-driven expression of *Cre* recombinase. (b) Flow cytometry analysis demonstrated that *trem1* deletion was specific to the monocytes of *trem1*$^{fl/fl}$Lyz2-*Cre*+ (*trem1*$^{-/-}$) mice in the blood during anemia; no deletion occurred in *trem1*$^{fl/wt}$Lyz2-*Cre*+ (*trem1*$^{+/-}$). Line diagrams show serial measurements of plasma cytokines (c) IFN-γ, (d) IL-1β, (e) TNF-α, (f) IL6, (g) MIP1α, (h) MIP1β, (i) MIP2 and (j) LIX in *trem1*$^{-/-}$ anemic-transfused mice (maroon lines) *vs* WT-anemic transfused littermates. *N*= 8 mice per group; **p*<0.05, ***p*<0.01, ****p*<0.001 *vs* WT-anemic-transfused at the respective time points.

Table 1.

Primer sequences used for reverse transcriptase real-time polymerase chain reaction for target genes.

Gene Name	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
IFN-γ	ATTACTACCTTCTTCAGCAACA	CGAATCAGCAGCGACTCC
IL1-β	AATCTATACCTGTCCTGTGTAA	GCTTGTGCTCTGCTTGTG
TNF-α	AGGACTCAAATGGGCTTTC	AGGTCTGAAGGTAGGAAGG
IL6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
MIP1-α	ATTGAGACCTCTGCCGACTT	TATTCCTCGTTTCATGATCCTCC
MIP1-β	CCACTTCCTGCTGTTTCTCTTA	CAAAGACTGCTGGTCTCATAGT
LBP	TGTGTAAGTGAGCAAGAGATG	GAACAGCAGTCCGATGAG
MD2	CGAAGCGTAAGGAAGTTC	GTATTGATGTATTCACAGTCTCT
CD14	CCTAATGCCACTCTGAAT	CACTTCTCTTCCTCCTAAC
RAGE	CCTTAGCTGGCACTTAGA	TCTCCTGGTCTCTTCCTT
HMGB1	TCGTGATAGTTGGATAAG	GTGTAAGGATTAGAAGGTA
TREM1	TCCTATTACAAGGCTGACAGAGCGTC	AAGACCAGGAGAGGAAACAACCGC
TLR2	AAGGAGGTGCGGACTTGTTTC	GAGCCAAAGAGCTCGTAGCA
TLR4	GACACTACCACAATAACC	TTCACCTCTGCCTTCACT
NFkB1	GTCCACTGTCTGCCTCTC	TCCTTCCTGCCCATAACC
NFkB2	CACAGAGATGGAGGAGTTG	GAGGCGAGTAAGAGTTGG
18s	TCCGATAACGAACGAGAC	CTAAGGGCATCACAGACC