Platelet Toll-like Receptor 4-related Innate Immunity Potentially Participates in Transfusion Reactions Independent of ABO Compatibility: An ex Vivo Study

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Research Article

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Abstract

**Purpose:** The role of platelet TLR4 in transfusion reactions remains unclear. This study analyzed platelet TLR4, certain DAMPs, and the effect of ABO compatibility on TLR4 expression after a simulated transfusion *ex vivo*.

**Methods:** Donor red blood cells were harvested from a blood bank. Recipient blood from patients undergoing cardiac surgery was processed to generate a washed platelet suspension. Donor blood was added to the washed platelets at 1%, 5%, or 10% (v/v). Blood mixing experiments were performed using four groups: 0.9% saline control group (n = 31); M, matched blood type mixing (n = 20); S, uncross-matched ABO type-specific mixing (n = 20); and I, ABO incompatible blood mixing (n = 20). Platelet TLR4 expression was determined after blood mixing. Levels of TLR4-binding DAMPs (HMGB1, S100A8, S100A9, and SAA) and that of LPS-binding protein and endpoint proteins (TNF-α, IL-1β, and IL-6) in the TLR4 signaling pathway were evaluated.

**Results:** The 1%, 5%, and 10% blood mixtures significantly increased TLR4 expression in three groups (M, S, and I; all P < 0.001) in a concentration-dependent manner. TLR4 expression did not significantly differ among the three groups (P = 0.148). HMGB1, S100A8, and S100A9 showed elevated levels in response to blood mixing; SAA, LPS-binding protein, TNF-α, IL-1β, and IL-6 did not.

**Conclusion:** Blood mixing may elicit innate immune responses by upregulating platelet TLR4 and DAMPs unassociated with ABO compatibility, suggesting that innate immunity through TLR4-mediated signaling may induce transfusion reactions.

The trial was retrospectively registered at Chinese Clinical Trial Registry (ChiCTR2100045606) with date of registration on 19 April 2021.

Introduction

Blood transfusions are critical interventions, particularly in patients undergoing hemorrhagic shock. However, despite significant advancements in the safety of blood transfusions, these procedures are still associated with important risks. The mechanisms of immune reactions during blood transfusion are unclear, specifically those of innate immune reactions. Transfusion reactions can result from transfusion with cross-matched blood, uncross-matched type-specific blood, and ABO incompatible blood [1]. Immune reactions to blood transfusion involve complex interactions among various soluble factors and immune cells, including platelets [1].

Platelets play an important role in hemostasis and thrombosis, and mediate innate immune responses [2]. They upregulate Toll-like receptor 4 (TLR4), serve as sentinels of the innate immune system, and are essential for stimulating adaptive immune responses [2–7]. We hypothesized that blood transfusion could induce platelet-mediated innate immune reactions caused by the interaction between TLR4 and damage-associated molecular patterns (DAMPs) [8]. To test this hypothesis, we assessed the levels of
TLR4, lipopolysaccharide (LPS)-binding protein, and certain DAMPs including high mobility group box 1 (HMGB1), S100 calcium-binding protein A8 (S100A8, MRP8, and calgranulin A), S100 calcium-binding protein A9 (S100A9, MRP14, and calgranulin B), and serum amyloid A (SAA) in a simulated cross-reaction ex vivo. Furthermore, pro-inflammatory cytokines downstream of TLR4 signaling, including TNF-α, IL-1β, and IL-6, were evaluated [8].

Methods

Reagents and flow cytometry

The following antibodies were used in this study: anti-TLR4-PE antibody (BD Biosciences, Franklin Lakes, NJ, USA), a monoclonal antibody targeting TLR4 expressed on the platelet cell surface; and anti-CD41a-FITC (BD Biosciences), a platelet-specific monoclonal antibody recognizing the platelet GPIIb/IIIa complex independent of activation. Furthermore, IgG1 κ-FITC and IgG1 κ-PE antibodies (BD Biosciences) were used to assess nonspecific binding. Thrombin (Sigma, St. Louis, MO, USA) was used for platelet stimulation (Fig. 1). The platelet wash buffer (20 mM HEPES, 145 mM NaCl, 9 mM Na₂EDTA, pH 7.4) and HEPES-buffered Tyrode’s solution (10 mM HEPES, 136.89 mM NaCl, 11.9 mM NaHCO₃, 1.61 mM KCl, 0.42 mM Na₂HPO₄, 1.05 mM MgCl₂, 5.6 mM glucose, pH 7.4) were prepared in our laboratory.

Blood sampling

This study was approved by the institutional review board of Tri-Service General hospital (TSGHIRB 1-102-05-014, TSGHIRB 1-107-05-015), and written informed consent was obtained from the participants prior to enrollment. Rh-negative patients were excluded from this study. Red blood cells (unwashed) with a hematocrit of 55–60% were acquired from the blood bank of our hospital and stored in a cold room at 2–4 °C. Recipient blood samples were obtained from patients scheduled for cardiac surgery. Blood samples were obtained from an arterial catheter before anesthesia induction (Fig. 2). All samples were treated with a 1:9 volume of 3.8% sodium citrate solution as an anticoagulant. Blood transfusion was simulated ex vivo by performing blood mixing. Blood mixing reactions were segregated into four groups: 0.9% saline control group; group M, matched blood type mixing group; group S, uncross-matched ABO type-specific mixing; group I, ABO-incompatible mixing group.

The primary outcome measure was the effect of blood mixing on platelet TLR4 expression in each of the three groups (M, S, and I) and the differences in TLR4 expression among the three groups (M, S, and I). The secondary outcome measure was the effect of blood mixing on the levels of LPS-binding protein and DAMPs including HMGB1, SAA, S100A8, and S100A9. Additionally, the levels of molecules downstream of TLR4 signaling, such as TNF-α, IL-1β, and IL-6, were evaluated in each of the three groups (M, S, and I) [8].
Flow cytometry analysis of TLR4 expression

Recipient whole blood was centrifuged at 37°C for 10 min at 200 × g. The upper phase (plasma) was carefully collected, whereas the lower phase (red cells) and interphase (buffy coat containing mainly leukocytes and a few platelets) were discarded. The upper phase was then centrifuged at 2,000 × g for 10 min and the remaining pellet was gently resuspended in platelet wash buffer. The suspension was centrifuged at 2,000 × g for 10 min to prepare washed platelets, which were resuspended in HEPES-buffered Tyrode's solution; the suspension was adjusted to a final platelet count of 150,000–450,000 platelets/μL. Thereafter, recipient washed platelets without or with 0.9% saline added at 1%, 5%, or 10% (v/v) were used as controls, respectively (Fig. 3). Consequently, for blood mixing reactions, donor red blood cells were immediately mixed with recipient washed platelets at 1%, 5%, or 10% (v/v) and incubated at 37°C for 5 min (Fig. 4).

Quantification of TLR4 expression in washed platelets

We investigated platelet TLR4 expression in mixed samples in the presence of thrombin (final concentration: 0.2 U/mL) by incubating the samples at an ambient temperature of 23–26 °C for 5 min. Thrombin is generated during tissue injury, such as cardiac surgery [9], and is a key component of the blood coagulation cascade and a potent stimulator of platelets. Thrombin is commonly used in sample preparation protocols for platelet analysis. To quantify TLR4 expression, the samples were stained with a saturating concentration of the anti-CD41a-FITC and anti-TLR4-PE monoclonal antibodies and incubated at 22–26 °C in the dark for 20 min. The samples were then fixed with 1% paraformaldehyde at 4 °C for 30 min and analyzed using flow cytometry. FITC-labeled IgG1κ and PE-labeled IgG1κ served as background controls. Individual platelets were identified through side scatter (granularity characteristics) and anti-CD41a-FITC immunofluorescence in a logarithmic-scaled dot plot. The results are expressed as the mean fluorescence intensity (MFI) of TLR4-PE expression, and reads from 10,000 platelets were collected for each sample (Fig. 3; Fig. 4).

ELISA analysis

We then assessed the levels of LPS-binding protein and of certain DAMPs that may interact with TLR4 after blood mixing. Donor red blood cells were immediately added to recipient whole blood at 1%, 5%, or 10% (v/v) and incubated at 37°C for 30 min. The mixed blood was then centrifuged at 37°C for 10 min at 100 × g. The upper phase (plasma) was carefully collected, whereas the lower phase (red cells) and interphase (buffy coat containing mainly leukocytes and a few platelets) were discarded. The plasma was analyzed to determine the concentrations of HMGB1 (Aviva Systems Biology, San Diego, CA, USA), S100A8 (Circulex, MBL, Nagano, Japan), S100A9 (Circulex, MBL), SAA (Abnova Co., Taipei, Taiwan), and LPS-binding protein (Aviva Systems Biology) using ELISA kits according to the manufacturer’s protocols.
Multiplex immunoassays

The plasma levels of pro-inflammatory cytokines, i.e., TNF-α, IL-1β, and IL-6, were assessed using multiplex immunoassay (ProcartaPlex Multiplex Immunoassay, Affymetrix eBioscience, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to compare demographic variables of the four groups (control, M, S, and I). Furthermore, ANOVA was performed to compare the levels of TLR4; LPS-binding protein; DAMPs including HMGB1, S100A8, S100A9, and SAA; and TLR4-regulated cytokines including TNF-α, IL-1β, and IL-6 in each of the three groups (M, S, and I) followed by Scheffé post-hoc test. Differences among the three groups (M, S, and I) over various concentrations were analyzed by two-way ANOVA. All tests were two-sided, with $P < 0.05$ considered as statistically significant. SPSS software (Version 20; SPSS, Inc., Chicago, IL, USA) was used for all analyses.

Results

No significant differences in demographic characteristics were observed among the four groups (Table 1). Figure 1 shows the results of flow cytometric analysis of platelet TLR4 expression. Figure 1 depicts fluorescence dot plots representing TLR4 expression in isotype controls (Fig. 1A), unstimulated platelets (Fig. 1B), thrombin-stimulated platelets (Fig. 1C), and thrombin-stimulated platelets mixed with 10% donor blood (Fig. 1D); Fig. 1E shows an overlay of the histograms of unstimulated platelets, thrombin-stimulated platelets, and thrombin-stimulated platelets mixed with 10% donor blood (Fig. 1E).

Table 1

Demographic characteristics of recipients in 0.9% saline control and groups M, S, and I.
### Effect of TLR4 expression after the addition of 0.9% saline

The 0.9% saline control groups were not exposed to red blood cells but were treated with 0.9% saline (Fig. 3). We examined the control groups to determine whether TLR4 expression levels could be determined in blood mixing experiments using a recipient washed platelet mixture containing 0.9% saline [1%, 5%, and 10% (v/v)]. The TLR4 expression levels did not significantly differ following stimulation by thrombin or the 0.9% saline control [1%, 5%, and 10% (v/v)] ($P = 0.892$, Fig. 3).

### Effect of TLR4 expression after the addition of donor red blood cells

The 1%, 5%, and 10% blood mixing induced a concentration-dependent significant increase in TLR4 expression levels in all three groups (M, S, and I; all $P < 0.001$; Fig. 4). For total TLR4 expression of all experimental groups, blood mixing induced increases of 147%, 288%, and 381% in TLR4 expression levels after 1%, 5%, and 10% mixing, respectively, compared to that in the control ($n = 60$, $P < 0.001$; Fig. 4). For group M, blood mixing induced increases of 143%, 308%, and 399% in TLR4 expression after 1%, 5%, and 10% mixing, respectively, compared to that in the control ($n = 20$, $P < 0.001$; Fig. 4). For group S, blood mixing induced increases of 157%, 291%, and 380% in TLR4 expression after 1%, 5%, and 10% mixing, respectively, compared to that in the control ($n = 20$, $P < 0.001$; Fig. 4). For group I, blood mixing induced increases of 142%, 265%, and 362% in TLR4 expression after 1%, 5%, and 10% mixing, respectively, compared to that in the control ($n = 20$, $P < 0.001$; Fig. 4). However, TLR4 expression levels did not significantly differ ($P = 0.148$; Fig. 4) among the three groups (M, S, and I).
Effect of DAMPs and LPS-binding protein in the plasma

HMGB1, S100A8, and S100A9 levels were significantly increased in response to blood mixing (Fig. 5). The total HMGB1 level was significantly increased by 122%, 200%, and 272% following 1%, 5%, and 10% mixing, respectively, compared with that in the control ($P < 0.001$; Fig. 5). The total S100A8 level was significantly increased by 104%, 114%, and 123% following 1%, 5%, and 10% mixing, respectively, compared to that in the control ($P < 0.001$; Fig. 5). The total S100A9 level was significantly increased by 115%, 138%, and 150% following 1%, 5%, and 10% mixing, respectively, compared with that in the control ($P = 0.012$; Fig. 5). However, the total SAA level was not significantly increased in each of the three groups (M, S, and I) ($P = 0.588$, $P = 0.999$, $P = 0.988$, respectively; Fig. 5).

Effect of pro-inflammatory cytokines downstream of the TLR4 signaling pathway in the plasma

The levels of total LPS-binding protein involved in TLR4 signaling ($P = 0.526$) and endpoint proteins including total TNF-α, IL-1β, and IL-6 were not elevated after blood mixing ($P = 0.998$, $P < 0.806$, $P < 0.87$, respectively; Fig. 6).

Discussion

This study showed that after ex vivo blood mixing, TLR4 expression levels were upregulated in platelets in the matched (M), uncross-matched ABO type-specific mixing (S), and ABO incompatible (I) groups. In addition to its crucial role in hemostasis and thrombosis, platelet TLR4 significantly contributes to amplifying inflammatory and immune responses [2]. This may also be true in the platelet TLR4-related transfusion immune and inflammatory responses [2]. Platelet TLR4 expression influences innate immunity [5, 7, 8, 10, 11], leading to an adaptive immune response [3–7] and significant inflammation [12] as observed in transfusion reactions. Thus, platelet TLR4 may serve as a pathophysiological link between innate immunity and transfusion reactions.

TLR4 recognizes DAMPs [13], which are associated with host cell components that are released upon cell damage [5, 14, 15]. We found that certain DAMPs, including HMGB1, S100A8, and S100A9, were elevated after blood mixing, which is corroborated by previous reports [16–20] on blood transfusions (Fig. 5). For example, first, red blood cell transfusion increases the susceptibility to lung inflammation through the release of HMGB1 and induces necroptosis in lung endothelial cells [19]. Second, stored human red blood cells contain soluble HMGB1, the levels of which are elevated during storage [16]. Third, salvaged blood analyses revealed sustained high levels of certain DAMPs, including S100A8 and S100A9. Above all, transfusion reactions may result from increased levels of TLR4 and certain DAMPs including HMGB1, S100A8, and S100A9 that bind with TLR4 (Fig. 5). Upregulation of TLR4 and certain DAMPs suggests that innate immunity through TLR4-mediated signaling can induce the transfusion reaction.
TLR4 expression upregulation and the elevated levels of some DAMPs (HMGB1, S100A8, and S100A9) were observed in response to blood mixing. However, this did not involve SAA and LPS-binding protein and did not lead to the release of pro-inflammatory cytokines downstream [8], such as TNF-α, IL-1β, and IL-6, after blood mixing (Fig. 6). These finding may be because of the following: first, LPS-binding proteins is a soluble protein that is synthesized by hepatocytes and found in the blood [21] that may not synthesized in ex vivo study and not to detected by ELISA. Second, negative regulators target multiple levels of TLR4 signaling, and several molecules, such as RP105 and SIGIRR, inhibit the initiation of this signaling cascade [8]. Other factors target molecules further downstream of TLR4 signaling through different mechanisms [8]. Third, these results may have occurred because of the short duration of blood mixing (30 min) during which time pro-inflammatory cytokines were not expressed; thus, the interaction between platelet TLR4 and certain DAMPs does not trigger this signaling pathway of pro-inflammatory cytokine release. Fourth, ex vivo blood mixing was not able to provide an adequate context in which to observe the complete range of platelet response to transfusion in vivo. Above all, although our results suggest that platelet TLR4 serves as a pathophysiological link between innate immunity and transfusion, the levels of pro-inflammatory cytokines downstream of TLR4 signaling, including TNF-α, IL-1β, and IL-6, were not increased remain unanswered.

Among the three groups (M, S, and I), we did not observe significant differences in TLR4 levels. The major variances among the three groups (M, S, and I) were the responses of antibodies recognizing transfused (foreign) antigens [22]. Human red blood cell membranes containing ABO system components are important in most blood transfusions. Individuals often produce antibodies (alloantibodies) against the alleles they lack within each system. Such antibodies are responsible for the most serious reactions to transfusions. However, ABO systems are not present on platelets [22]. In addition, surface antigens on red blood cells include Duffy, Kell, Kidd, MNS, and P systems, which are not present on platelets [22]. We found no differences in platelet TLR4 expression among the three groups (M, S, and I), which implies that reactions between antibodies and antigens did not play a role in inducing TLR4 expression.

TLR4 ligands recognize not only DAMPs but also pathogen-associated molecular patterns (PAMPs) [5, 14, 15, 23], such as circulating LPS (endotoxin), which are associated with microbial pathogens [24]. PAMP signaling was less likely to participate in this response for the following two reasons: first, during blood mixing, PAMPs such as circulating LPS from microbial pathogens were disregarded, as the patients did not have a pathogenic infection; second, after blood mixing, the levels of LPS-binding protein, which binds with PAMPs but not DAMPs, did not increase (Fig. 6) [14, 15]. Therefore, blood mixing may initiate platelet TLR4 expression, which may trigger the innate immune system, likely independent of PAMPs.

The rationale for selecting patients undergoing cardiac surgery in this study was as follows: first, blood transfusion is routinely required during cardiac surgery, and the preparation of surgery routinely requires cross-match testing of blood at least one day before surgery. Therefore, cross-matched red blood cells were available routinely and sent to operation room from the blood bank before anesthesia for us to obtain donor red blood cells. The red blood cells also used for heart-lung machine priming or stored until transfusion later; second, patients undergoing cardiac surgery require arterial catheterization for
aggressive hemodynamic monitoring. The arterial catheterization also used for recipient blood sampling before anesthesia induction and operation of skin incision prevents contamination due to anesthetics and tissue damage, which may be a confounding factor affecting platelet function and activation (Fig. 2). Red blood cells from groups S and I were also obtained from the blood bank for other major surgeries in this hospital.

Our blood mixing procedure is clinically relevant. In this study, we performed ex vivo mixing of donor and recipient blood, which is similar to the cross-matching procedure. Cross-matching is performed prior to blood transfusion to determine whether donor blood is compatible (or incompatible) with recipient blood. The mean body weight of subjects in this study was 66.8 kg; thus, the calculated total blood volume was approximately 4620 mL (estimated 7% of body weight). We used mixtures of 1–10%, which were equivalent to approximately 46.8 to 467.6 mL, respectively; these volumes are commonly used in clinical transfusions.

This study had two limitations. First, the baseline levels of HMGB1, S100A8, S100A9, SAA, LPS-binding protein, TNF-α, IL-1β, and IL-6 were not detected in the donor red blood cells. However, donor red blood cells were added to recipient whole blood at 1%, 5%, or 10% (v/v). The donor red blood cells have a hematocrit level of 55–60% and were standardized by our blood bank center. Thus, they would have contained less plasma than recipient whole blood. Therefore, the plasma volume of our donor red blood cells was far smaller than that of recipient whole blood. Second, we primarily used an ex vivo model because we included group I (ABO incompatible blood mixing), which could be harmful to patients if conducted in vivo. Therefore, the in vivo host innate immune responses were not evaluated. Platelets are not only directly involved in immune defense, but also assist and regulate several functions of innate immune cells. Platelets have also been shown to participate directly in the modulation of immune cell function by physically tethering to them or releasing platelet-derived microvesicles, lipid mediators, nucleosides, mitochondrial DNA, growth factors, cytokines, and chemokines. Platelets and their releasates have broader effects on differentiation, migration, phagocytosis, microbicidal activity, formation of extracellular traps, pathogen clearance, and cytokine response of innate immune cells [24].

**Conclusions**

Platelet TLR4 functions at the crossroads of thrombosis and the innate immune response. We found that allogeneic blood mixing may modulate the innate immune response by upregulating platelet TLR4 and DAMPs, including HMGB1, S100A8, and S100A9, which may bind to TLR4, thus suggest that platelet TLR4 links transfusion and innate immunity in blood mixing reactions. Considering the large number of circulating platelets, the potential interaction between platelet TLR4, and DAMPs and the induction of innate immune responses leading to transfusion reaction is possible. However, because TLR4 downstream pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were not detected, it remains unclear whether TLR4 signaling leads to a transfusion reaction under in vivo conditions. This upregulation of TLR4 expression was not associated with ABO compatibility. These findings suggest that TLR4 contributes to transfusion reactions that are unrelated to antibodies against red blood cell antigens.
Whether platelet TLR4 can be considered a novel prophylactic and therapeutic target in transfusion reactions or a new target to modulate innate immunity remains further studies.

**Declarations**

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**Conflicts of interest/Competing interests:**

The authors have no conflicts of interest or financial ties to disclose.

**Availability of data and material:**

The datasets generated during and/or analysed during the current study are available in the Mendeley Data repository, [https://data.mendeley.com/datasets/4fhvtdmjsx/1](https://data.mendeley.com/datasets/4fhvtdmjsx/1)

**Code availability:**

Not applicable

**Authors’ Contributions:**

CST and GSH conception and design of the work; CST and GSH designed and executed the experiments; CST, MHH, YCH, and GSH provided acquisition, analysis and interpretation of data; CST and GSH wrote the manuscript; CST, MHH, YCH, and GSH reviewed, edited and approved the manuscript.

**Compliance with Ethical Standards:**
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

**Consent to participate and consent for publication:**

Informed consent was obtained from all individual participants included in the study. The study was approved by the institutional review board of Tri-Service General hospital (TSGHIRB 1-102- 05-014, TSGHIRB 1-107-05-015).

**References**


**Figures**
Flow cytometric analysis of platelet Toll-like receptor 4 (TLR4) expression. Individual platelet events are identified by their characteristic side-scatter properties (granularity; x-axis) and positive labeling using platelet-specific monoclonal antibodies (CD41a-FITC; y-axis). Dot plot of the fluorescence of isotype control (A), unstimulated platelets (B), thrombin-stimulated platelets (C), and thrombin-stimulated + 10% donor blood (D). 

Figure 1
platelets with 10% donor blood (v/v) (D). Histogram of the fluorescence of unstimulated (control) and thrombin-stimulated platelets (TLR4-PE; x-axis) (E). MFI, mean fluorescence intensity.

Figure 2

Key steps of general anesthesia and cardiac surgery performed in this study. First, essential hemodynamic monitoring was conducted. Second, cross-matched red blood cells were sent to the operation room. Then, arterial catheterization was performed to obtain donor red blood cells and recipient blood samples. Anesthetics were administered, and skin incision was performed. Blood sampling before anesthesia induction and skin incision can theoretically eliminate confounding factors caused by anesthetics and tissue damage-related factors in the blood circulatory system.
Figure 3

Effect of Toll-like receptor 4 (TLR4) expression after 0.9% saline was mixed with washed platelets (n = 31; n = 10, group M; n = 10, group S; n = 11, group I). MFI, mean fluorescence intensity; vol, volume; WP, washed platelets. Data are presented as the mean ± SD.
Figure 4

Effect of Toll-like receptor 4 (TLR4) expression on matched blood type mixing (Group M), uncross-matched ABO type-specific mixing (Group S), and ABO incompatible blood mixing (Group I) after mixing donor red blood cells and washed platelets. MFI, mean fluorescence intensity; RBCs, red blood cells; vol, volume; WP, washed platelets. Data are expressed as the mean ± SD. *P < 0.05.
Figure 5

Effect of DAMPs (HMGB1, S100A8, S100A9, and SAA) in the plasma prepared from mixing donor red blood cells and recipient whole blood on total blood mixing, matched blood type mixing (Group M), uncross-matched ABO type-specific mixing (Group S), and ABO incompatible blood mixing (Group I). HMGB1, high mobility group box-1; SAA, serum amyloid A; RBCs, red blood cells; vol, volume; WP, washed platelets. Data are expressed as the mean ± SD. *P < 0.05.
Figure 6

Effect of LPS-binding protein and pro-inflammatory cytokines in the plasma, prepared from mixing donor red blood cells and recipient whole blood, on matched blood type mixing (Group M), uncross-matched ABO type-specific mixing (Group S), and ABO incompatible blood mixing (Group I). RBCs, red blood cells; vol, volume; WP, washed platelets. Data are expressed as the mean ± SD. *P < 0.05.