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Complement deposition on the surface of RBC after trauma serves a biomarker of moderate trauma severity: A prospective study

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Abstract

Background: Activation of the complement system and complement deposition on red blood cells (RBCs) contribute to organ damage in trauma. We conducted a prospective study in subjects with traumatic injuries to determine the pattern of complement deposition on RBC and whether they are associated with clinical outcomes.

Method: A total of 124 trauma patients and 42 healthy controls were enrolled in this prospective study. RBC and sera were collected at 0, 6, 24 and 72 hours from trauma patients and healthy controls during a single draw. Presence of C4d, C3d, C5b-9, phosphorylation of band 3 and production of nitric oxide were analyzed by flow cytometry.

Results: RBC from trauma patients at all time points up to 24 hours displayed significantly higher deposition of C4d on their RBC membrane as compared to healthy donors. Incubation of normal RBC with sera from trauma patients resulted in significant increase of C4d deposition (at 0, 6, 24 and 72 hours), C5b-9 deposition (at 0 and 6 hours), phosphorylation of band 3 (at 0 and 24 hours), and nitric oxide (NO) production up to 24 hours compared to sera from healthy subjects. Deposition of C4d and C5b-9 in patients with an ISS of 9 and above remained elevated up to 72 hours.

Conclusions: Our study demonstrates that the presence of C4d, C3d, and C5b-9 on the surface of RBC is linked to increased phosphorylation of band 3 and increased production of nitric oxide.

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Conflict of interest: None

Deposition of C4d and C5b-9 decreased faster over course of 3-day study in subjects with ISS less than 9.

Keywords

Complement; trauma; C4d; C3d; C5b-9; band 3; nitric oxide

Introduction

The consequences of trauma cannot be ignored with over 10 million motor vehicle collisions occurring every year with enormous clinical challenge (1–3). Despite significant advances in injury prevention, prehospital resuscitation strategies, and modern intensive care, trauma remains the leading cause of death resulting from accidents or unintentional injuries for those less than 50 years old and accounts for one out of about every 20 deaths in the United States. (4–6).

Although trauma involves anatomical injury that leads to hemorrhage, there are also important pathophysiologic sequelae that compromise organ function that are not directly affected by initial injury. The origin of these secondary manifestations remains incompletely understood (7). Survival of patients after severe tissue trauma first requires an adequate surgical management. However, equally important is the rational therapeutic approach of complications arising from the metabolic and immunologic disturbances. Data suggest that the innate immune response (including the complement system, coagulation cascade, and neutrophils) which intends to control the injury paradoxically confers collateral damage leading to manifestations from multiple organs (8).

Trauma can affect any individual at any location and at any time over a lifespan. The instant activation of complement systems and interacting platelets are rapidly activated after disruption of micro- and macro-barriers, which results in a stemming of hemorrhage and protection against invading bacteria (9). Activation of the complement system occurs through three distinct pathways: the Classical pathway, the Lectin Pathway and the Alternative pathway. In addition, the complement system can be activated via the coagulation system and most notably from the generation of thrombin (10). All three activation pathways lead to the generation of C3 convertase which then cleaves C3 to generate C3a and C5a whereas thrombin acts directly on C3 and C5 to generate C3a and C5a respectively. All three complement activation pathways and the coagulation pathway subsequently lead to generation of the membrane attack complex C5b-9 (MAC). However, only the classical pathway leads to the generation of C4 via C1q (11). Together, complement activation and the proinflammatory cytokine storm are two of the main triggers of pathology which occurs in patients after trauma. They collectively choreograph a systemic inflammatory response syndrome (SIRS) associated with acute respiratory distress syndrome and multiple organ failure (12). In other situations where complement is profusely activated, such as in systemic lupus erythematosus (SLE), complement fragments deposit on the surface of red blood cells (RBC). C4d and C3d are the stable remnants of C4 and C3 respectively, and they remain on the surface of RBC, as demonstrated in patients with SLE, for a prolonged period of time. (13, 14) C4d and C3d deposition limits the deformability of

RBC while promoting nitric oxide production (15). RBC membrane deformability is linked to the phosphorylation status of membrane proteins such as β -spectrin or band 3 (16, 17). We have previously reported that C4d decorates the surface of RBC and possibly limits their ability to deform and pass through capillary-size microchannels and increases the production of NO in patients suffering from trauma (7). It has been reported that human RBC express an active and functional endothelial-type NO synthase (eNOS) which is present in the membrane and in the cytoplasm (18). In RBC, mechanical stress (shear stress) stimulates NO generating mechanisms and Ca^{2+} is important in this process (19). Nitric oxide is a free radical gas produced from arginine by nitric oxide synthase and is an important vasodilator which acts on vascular endothelium and underlying vascular muscle and is an important inhibitor of platelet aggregation (20).

Understanding the pattern of complement deposition following trauma is crucial to the development of proper interventional strategies to accompany or replace conventional treatment methods. Human RBC have an average life span in the circulation of approximately 120 days (21). However, the various activation products of complement have diverse half-lives *in vivo*. For the analysis of complement deposition on RBC, it is essential to measure activation products of choice in a similar way. Due to rapid receptor binding, the biologically highly active and important C5a fragment has a half-life of approximately 1 min (22) and is difficult to detect in samples obtained *in vivo*, whereas the various C3 activation products are readily detectable due to half-lives of a few hours (23). The half-life of SC5b-9 is 50 to 60 min (24). SC5b-9, in contrast to C3 activation products, is relatively stable *in vitro* and is a reliable indicator of terminal pathway activation. Complement activation products are usually present in only trace amounts *in vivo*, but they are rapidly generated *in vitro* (25).

Due to the longevity of RBC survival *in vivo* and the short circulating half-lives of complement split products, we applied two strategies to analyze complement deposition on RBC. First, the collected samples were stored properly to avoid *in vitro* activation. Second, RBC from universal donors were incubated with sera isolated from whole blood of trauma patients that were collected upon admission to the emergency department (0 hour), and 6, 24 and 72 hours later. We hypothesized that traumatic injury activates the complement system and complement activation products deposit on the surface of RBC and could limit RBC function differently at various time intervals after trauma. The aim of this prospective study was to investigate serial changes in complement deposition after traumatic injury in patients and seek correlations with clinical outcomes.

Materials and Methods

Participants:

This prospective, observational study was approved by the institutional Review Board of Beth Israel Deaconess Medical Center, and was carried out with written informed consent. A total of 124 patients who visited the emergency department at Beth Israel Deaconess Medical Center for trauma from September 2015 to June 2018 were enrolled non-consecutively. Trauma patients were 18 years and older, and were admitted for long bone fracture or organ injury. Blood tests were performed as required for their care. Blood

samples were collected at the time patients were enrolled in the study in the emergency department (0 hour) and again at 6, 24, and 72 hours post-enrollment. Patients diagnosed with infection or autoimmune diseases were excluded from the study. None of the patients had received a transfusion prior to sampling. Clinical data were recorded including age, sex, ethnicity, mechanism of injury, and existing injury. Injury Severity Score (ISS) was calculated. Healthy donors (n=42) were recruited from the Division of Rheumatology and Emergency Department at Beth Israel Deaconess Medical Center after written informed consent. They did not have any apparent illness.

Antibodies and reagents

Primary antibodies were anti-C4d monoclonal antibody (Quidel, San Diego, CA) (murine anti-human monoclonal C4d, dilution 1:250), anti-C3d monoclonal antibody (Quidel, San Diego, CA) (murine anti-human monoclonal C3d, dilution 1:25), anti-C5b-9 polyclonal antibody (Abcam, Cambridge, MA) (rabbit polyclonal to C5b-9, dilution 1:100) and IgG1k-isotype control (BioLegend, San Diego, CA). Secondary antibody was Alexa Fluor 488-conjugated rabbit anti-mouse IgG and donkey anti-rabbit IgG (Life technologies, Grand Island, NY) (dilution 1:100). Other reagents were as follows: Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} (HBSS⁺⁺) (Life technologies), IgG-free bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO), DAF-FM diacetate (Life technologies) and eosin-5-maleimide (Life technologies).

RBC preparation and incubation with sera

Blood was collected in heparin-lithium tubes for RBC and for serum in serum-separator tubes. For serum separation the tubes were centrifuged for 10 min at 1300 xg and at room temperature. Serum was collected in polypropylene plastic test tubes and was stored at -80°C . Heparinized blood was washed in HBSS⁺⁺ before use. On the day of the experiment for serum studies, serum samples were thawed from various trauma patients and healthy donors and were used for different measurements under identical conditions. Blood from healthy universal donors (type O, Rh negative) was obtained in heparin-lithium tubes and washed with HBSS⁺⁺. One μl of RBC pellet was resuspended in 80 μl of HBSS⁺⁺, and incubated with 20 μl of serum from trauma patients or normal donors for 15 minutes at 37°C , and then washed in HBSS⁺⁺ with 0.5% IgG-free BSA (0.5% BSA/HBSS⁺⁺).

Flow cytometry

RBC were incubated for 20 minutes with a primary antibody in 0.5% BSA/HBSS⁺⁺ at room temperature, washed and incubated for another 20 minutes with a fluorescence-conjugated secondary antibody directed against the primary antibody at a dilution recommended by the manufacturer. RBC were then washed and analyzed by FACScan (BD Biosciences, San Jose, CA). At least 10,000 events in each sample were acquired and recorded, and analyzed by FlowJo (version 10 software; Tree Star, Ashland, OR).

Eosin-5-maleimide staining

RBC from healthy universal donors were incubated in the presence of Ca^{2+} and Mg^{2+} with 20% trauma or control sera for 5 minutes at 37°C , and then incubated for 15 min with

eosin-5-maleimide at a final concentration of 0.1 mg/ml. RBC then were washed 3 times with HBSS⁺⁺ and 0.5%BSA/HBSS⁺⁺ and analyzed by flow cytometry.

Measurement of NO production

RBC from healthy universal donors were preloaded with DAF-FM diacetate in BSA-free HBSS⁺⁺ and incubated for 30 minutes at 37°C. RBC were washed to remove excess and un-cleaved intracellular DAF-FM diacetate probe. Cells were then resuspended in HBSS⁺⁺ with 20% trauma or control serum for 15 minutes at 37°C. RBC were washed and the fluorescence intensity associated with intracellular NO production was recorded by FACScan using the FL-1 channel.

Statistical analysis

Both SAS (version 9.4 for Windows) and Graphpad Prism (version 6 for Windows) were used for statistical analysis. We fit regression models using generalized estimating equations (GEE) methods to account for correlated repeated measures within patients. Based on these models, we compared logarithmic transformed outcome measures at 0, 6, 24 and 72 hours for trauma patients to healthy controls. Adjustment for multiple comparisons was done by Sidak. An adjusted value of $P < 0.05$ was considered statistically significant. Correlation between the ISS and the amount of C4d, C3d, C5b-9 deposition, Band 3 phosphorylation and NO production was assessed by Spearman correlation coefficient (r_s) and significance (p).

Results

Patient characteristics:

124 patients were included in this study. The patient group consisted of 53 (43%) women and 71 (57%) men with mean age of 54 (\pm 21) years (Table 1). Most of the patients presented with blunt injury resulting from motor vehicle crash or fall. The mean value of ISS was 12.3(\pm 10.2) and [3–34] range.

Deposition of C4d on RBC from patients with trauma

We determined the amount of C4d deposition on RBC from trauma patients over time and normal subjects by flow cytometry as shown in Figure 1A (representative experiment). We used 102 samples from trauma patients (n, 0 hour =102, 6 hour =24, 24 hour =35 and 72 hour =19) and 30 samples from healthy donors. RBC from trauma patients displayed significantly higher deposition of C4d on their membrane as compared to RBC from healthy donors at all time points up to 24 hours ($p < 0.05$). There were no significant differences between healthy donors and trauma patients at 72 hours, suggesting that C4d levels returned to basal levels before 72 hours (Figure 1B and Table 2). We noted a significant correlation between the ISS and the amount of C4d deposited on the surface of RBC ($r_s=0.25$, $p=0.010$). Supplementary figure 1 shows pattern of deposition of C4d on RBC according to ISS that was grouped in 8 subgroups (ISS 3 – 6, 7 – 10, 11 – 14, 15 – 18, 19 – 22, 23 – 26, 27 – 30 and 31 – 34).

C4d deposition on healthy RBC incubated with sera from trauma patients

We developed a method to assess whether C4d in sera from trauma patients decorated RBCs. Incubation of RBC from universal donors (type O, Rh negative) with sera from trauma patients resulted in complement C4d deposition on the surface of RBC (Figure 2A). Incubation of normal RBC with sera from trauma patients (n, 0 hour =100, 6 hour =18, 24 hour =31 and 72 hour =14) resulted in significant increase of C4d deposition at all time points up to 72 hours compared to sera from healthy controls (n=37) ($p < 0.05$) (Figure 2B and Table 2). We stratified patients into two groups according to ISS (below and above 9) to detect patterns of C4d deposition. As shown in Figure 2C, deposition of C4d in patients with an ISS of 9 and above remained elevated up to 72 hours when compared to patients with an ISS below 9. Supplementary figure 2 shows pattern of deposition of C4d according to ISS that was grouped in 8 subgroups (ISS 3 – 6, 7 – 10, 11 – 14, 15 – 18, 19 – 22, 23 – 26, 27 – 30 and 31 – 34).

C3d deposition on healthy RBC incubated with sera from trauma patients

Next we looked at the deposition of C3d using the method described for C4d whereby we incubated RBC from universal donors (type O, Rh negative) with sera from trauma patients or healthy donors. Incubation of RBC with sera from trauma patients at 0 hour (n=104) resulted in higher C3d deposition on the surface of RBC when compared to sera from healthy controls (n=36) but this increase were not statistically significant ($p=0.1421$). We also did not observe a significant difference between trauma patients and healthy donors at the later time points of 6, 24 and 72 hours (Figure 3 and Table 2). Supplementary figure 3 shows pattern of deposition of C3d according to ISS that was grouped in 8 subgroups (ISS 3 – 6, 7 – 10, 11 – 14, 15 – 18, 19 – 22, 23 – 26, 27 – 30 and 31 – 34).

C5b-9 deposition on healthy RBC incubated with sera from trauma patients

Deposition of C5b-9 was also analyzed using the same method as described for C3d. Incubation of normal RBC with serum obtained from blood of individual trauma patients at 0 hour (n=84), 6 hours (n=18), 24 hours (n=31) and 72 hours (n=14) showed a significant increase in C5b-9 deposition in samples up to 6 hours compared to sera from healthy controls (n=34) ($p=0.0026$ for 0 hour and $p=0.0034$ for 6 hours) (Figure 4A and Table 2). Moreover, there was a significant correlation between the ISS and C5b-9 deposition on surface of RBC ($r_s=0.36$, $p=0.001$). As shown in Figure 4B, deposition of C5b-9 in patients with an ISS of 9 and above remains elevated up to 72 hours when compared to patients with an ISS below 9. Supplementary figure 4 shows pattern of deposition of C5b-9 according to ISS that was grouped in 8 subgroups (ISS 3 – 6, 7 – 10, 11 – 14, 15 – 18, 19 – 22, 23 – 26, 27 – 30 and 31 – 34).

Phosphorylation status of band 3: To determine whether band 3 becomes phosphorylated in RBC exposed to trauma sera, we treated universal donor RBC exposed to trauma (n=97) or control (n=35) sera with eosin-5-maleimide, a reagent which binds to lysine 430 on the extracellular loop of band 3 and is known to indicate tyrosine phosphorylation. Binding of eosin-5- maleimide to RBC has been used previously to estimate the phosphorylation levels of band 3 (16, 26). Here we used eosin-5-maleimide to

estimate the tyrosine phosphorylation levels of band 3. Serial samples of sera from trauma patients at various time points (n, 0 hour =97, 6 hour =18, 24 hour =31 and 72 hour =14) showed a significant increase in the phosphorylation of band 3 at 0 and 24 hours compared to sera from healthy controls (n=35) (Figure 5 and Table 2). The phosphorylation of band 3 significantly correlated with deposition of C4d ($r_s=0.210$, $p=0.043$) and C5b-9 ($r_s=0.469$, $p<0.0001$). Supplementary figure 5 shows pattern of phosphorylation status of band 3 according to ISS that was grouped in 8 subgroups (ISS 3 – 6, 7 – 10, 11 – 14, 15 – 18, 19 – 22, 23 – 26, 27 – 30 and 31 – 34).

Induction of the NO production: Since we previously observed that exposure of RBC to trauma serum results in increased intracellular Ca^{2+} concentrations (7), we asked whether it also results in increased NO production. Incubation of normal RBC with sera from trauma patients (n, 0 hour =104, 6 hour =18, 24 hour =31 and 72 hour =14) resulted in significant increase of NO production at 0, 6 and 24 hours compared to sera from healthy controls (n=38) (Figure 6A and Table 2). There was no significant difference between trauma and healthy donor serum at 72 hour indicating that NO production returns to basal levels by that time point. Supplementary figure 6 shows NO production according to ISS that was grouped in 8 subgroups (ISS 3 – 6, 7 – 10, 11 – 14, 15 – 18, 19 – 22, 23 – 26, 27 – 30 and 31 – 34). Increased NO production resulted from complement deposition on RBC surfaces since heat inactivation of patient sera ($55^{\circ}C$ for 30 minutes) eliminated their ability to increase NO production by RBC (Figure 6B).

Discussion

In this prospective study we report increased deposition of complement split products (C4d and C5b-9) and enhanced levels of associated molecules (band 3 and NO) in trauma patients. Moreover, we find that the level of these molecules changes over time after trauma and declines more rapidly in patients with less severe (ISS less than 9) trauma. Our findings also confirmed a significant correlation between ISS and deposition of C4d and C5b-9 on RBC.

Identification of complement coated RBC from trauma patients while informative may not offer strong prognostic value because RBC are live long in the circulation and cells decorated with complement will be around for several days or weeks. However, testing the ability of sera to coat RBC from universal donors at any time during trauma offers a good estimate of the rate of complement activation and generation of short-lived complement split products. As shown by our data, C4d coated RBC is significantly greater compared to controls at admission. This is similarly observed when serum from trauma patients obtained at admission is used to coat C4d and C5b-9 on universal donor RBC. In contrast, serum from trauma patients obtained at later time points (6, 24 and 72 hours) revealed that elevation of C5b-9 occurs only transiently (able to coat RBC at the zero hour and six hours), C4d is elevated in the circulation over a longer time period (0 through 24 hours) and unlike C5b-9, C4d can coat RBC at all times tested (0 through 72 hours). We speculate that if RBC sampling was continued at later time points post admission, there would likely be a continued significant difference between trauma patients and controls. Indeed, sera from patients who have higher ISS have a sustained ability to coat RBC for a longer period of

time. It is clear from our data that this differential analysis may provide prognostic value when correlated with the level of injury (ISS) upon admission to the Emergency Department and thereafter.

The activation of the complement pathway has been characterized by deposition of split products initiated at the early phase of trauma and may remain on RBC from hours to days. To date, there is little data regarding the effects of the non-inflammatory complement by-products generated during complement activation after trauma and its time course on circulating cell function including RBC. In this study we show that C4d, C3d and C5b-9, deposits on the surface of RBC and enhances NO production. These functional effects are likely due to the fact that C4d deposition on the surface of RBC promotes a significant RBC Ca^{2+} influx (7) that enhances phosphorylation of band 3. Similar deposition of C4d was recorded in RBC from patients with SLE in whom complement activation is prominent (15). The enhanced phosphorylation of band 3 at 0 hour returned to basal levels at 6 hours and again increased at 24 hours showing a biphasic response. This may occur due to factors other than complement that might also affect RBC membrane deformability.

The functional consequences of the deposition of complement on the surface of RBC cannot be overstated. In order for RBC to perform their primary function, that is to deliver oxygen to tissues, they must pass through narrower ($<8 \mu\text{m}$ in diameter) capillaries. To do this, RBC must be able to deform (27, 28). Hypoxia may occur if deposition of C4d, C3d and C5b-9 renders RBC unable to deform and reach tissues. During normal conditions, RBC-generated NO is a key factor in the local regulation of vasomotor tone and microvascular flow resistance, by interacting directly with endothelial cells and indirectly with vascular smooth muscles (29). Nitric oxide derived from eNOS present in RBC also directly regulates and maintains RBC deformability (18, 30). In our study we demonstrated that NO production increases under trauma conditions and that may be due to increased complement deposition that decreases RBC deformability (7). We also found a significant correlation between phosphorylation of band 3 and NO production that further confirms the RBC cytoskeletal stiffness that may limit the deformability of RBC (31–33).

Our studies have certain limitations including the fact that the studied cohort was quite diverse in terms of origin of trauma (bone versus soft organs). While we limited the effect of important comorbidities by excluding patients with significant comorbidities from the study, the data about plausible confounding variables such as smoking, substance abuse, and medications were not collected. In addition, we were unable to recruit exact age-, ethnicity-, and sex- matched healthy individuals, and hence these parameters show differences between trauma and control groups. Most of the patients with minor injury who visited the emergency department at Beth Israel Deaconess Medical Center had low ISS scores. We were unable to collect a larger number of samples with high ISS scores. We believe that due to the low number of samples with high ISS, many complement molecules did not significantly correlate with ISS. Time 0 in our study was considered the time point of arrival and evaluation in the Emergency Department. Although all study parameters and sample handling were controlled thereafter, the elapsed time between the time the trauma occurred and the time of collection of the first sample was neither recorded nor considered.

Conclusions:

We conclude that C4d, C3d, and C5b-9 decorates the surface of RBC which enhances the phosphorylation of band 3 and increases the production of NO in various types of trauma for at least 72 hours. This study confirmed the significant correlation between injury severity score (ISS) and deposition of C4d and C5b-9 and that subject with severe trauma (ISS more than 9) maintain high deposition of complement components for at least 72 hours. Complement deposition on RBC alters their ability to deform and travel through capillaries and exchange gases in tissues and thus contributes to trauma-associated morbidity and mortality. Our prospective study strengthens the argument that complement activation and deposition on RBC may serve as a biomarker of trauma severity and complement inhibiting drugs and biologics should have a place in the treatment of subjects suffering trauma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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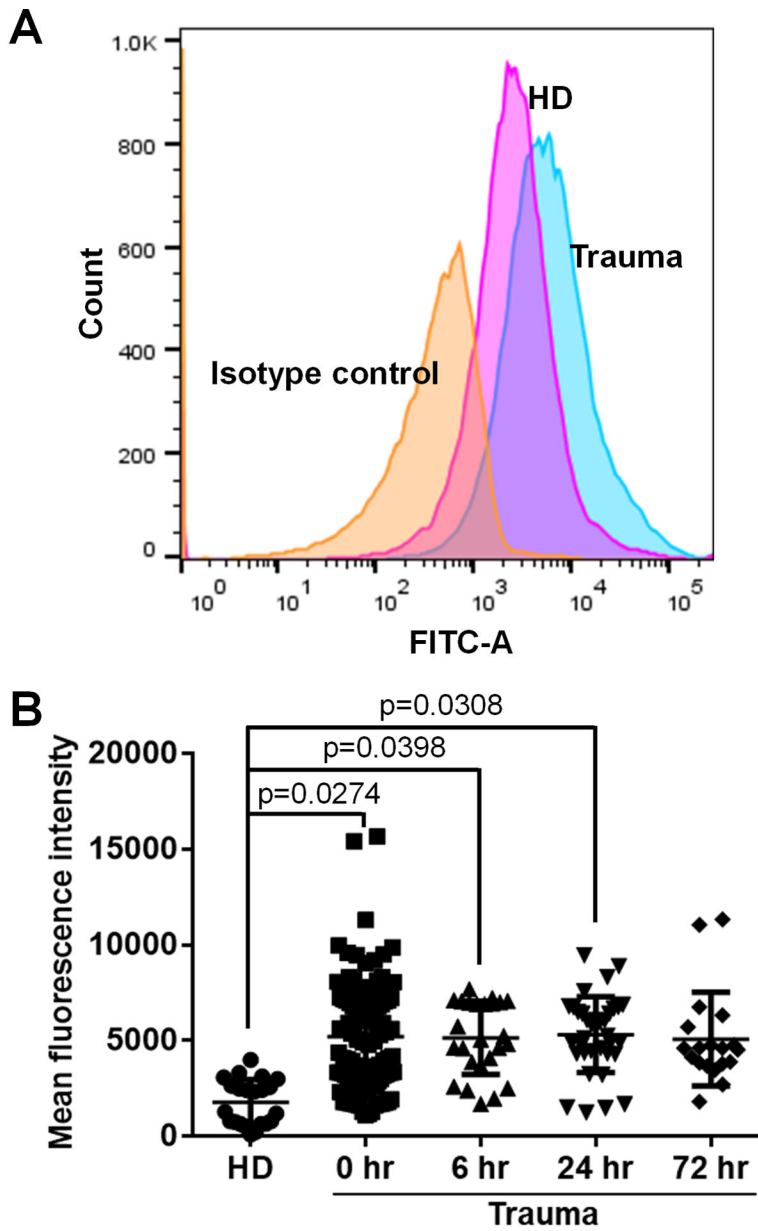


Figure 1. Increased C4d deposition on the surface of RBCs from trauma patients. Deposition of C4d on the surface of RBCs from trauma patients and healthy donors was measured by flow cytometry and mean fluorescence intensity. **A**, Representative flow cytometry experiment. **B**, we used samples from 102 trauma patients (n, 0 hour =102, 6 hour =24, 24 hour =35 and 72 hour =19) and 30 samples from healthy donors (HD). The mean fluorescence intensity [Mean \pm SD (min - max)] of C4d deposition on the surface of RBCs was [2278 \pm 1504 (94 - 5631)] for healthy donors, [5325 \pm 2941 (1113-15683)] for 0 hour, [5254 \pm 1862 (1698 - 7687)] for 6 hour, [5306 \pm 1988 (1245 - 9462)] for 24 hour and [5074 \pm 2444 (1802 -11346)] for 72 hour patients.

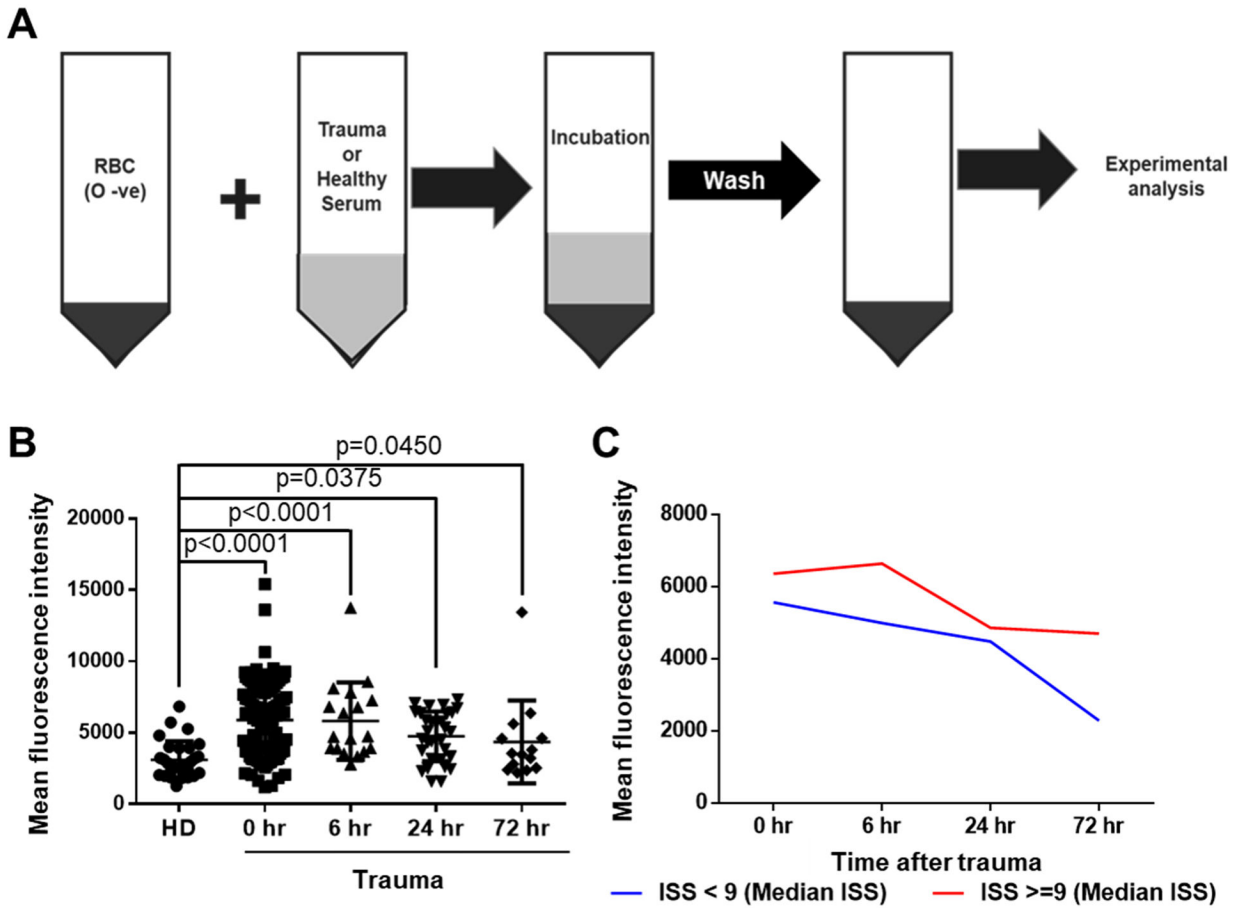


Figure 2. Deposition of C4d on the surface of healthy control RBCs (type O, Rh negative) incubated with sera from trauma patients or healthy control donors.

A, incubation of RBC from universal donors (type O, Rh negative) with sera from trauma patients or healthy donors. **B**, the C4d deposition on RBC from trauma patients over time compared to normal controls by flow cytometry and mean fluorescence intensity. We used samples from 100 trauma patients (n, 0 hour =100, 6 hour =18, 24 hour =31 and 72 hour =14) and 37 samples from healthy donors (HD). The mean fluorescence intensity [Mean \pm SD (min - max)] of C4d deposition on the surface of healthy control RBCs was [3233 \pm 1610 (1197 – 9215)] for healthy donors, [6071 \pm 2578 (1647–15404)] for 0 hour, [5817 \pm 2701 (2795–13764)] for 6 hour, [4753 \pm 1741 (1585 –7331)] for 24 hour and [4361 \pm 2903 (2206 –13450)] for 72 hour patients. **C**, deposition of C4d in patients with an ISS of 9 and above remains elevated up to 72 hours when compared to patients with an ISS below 9.

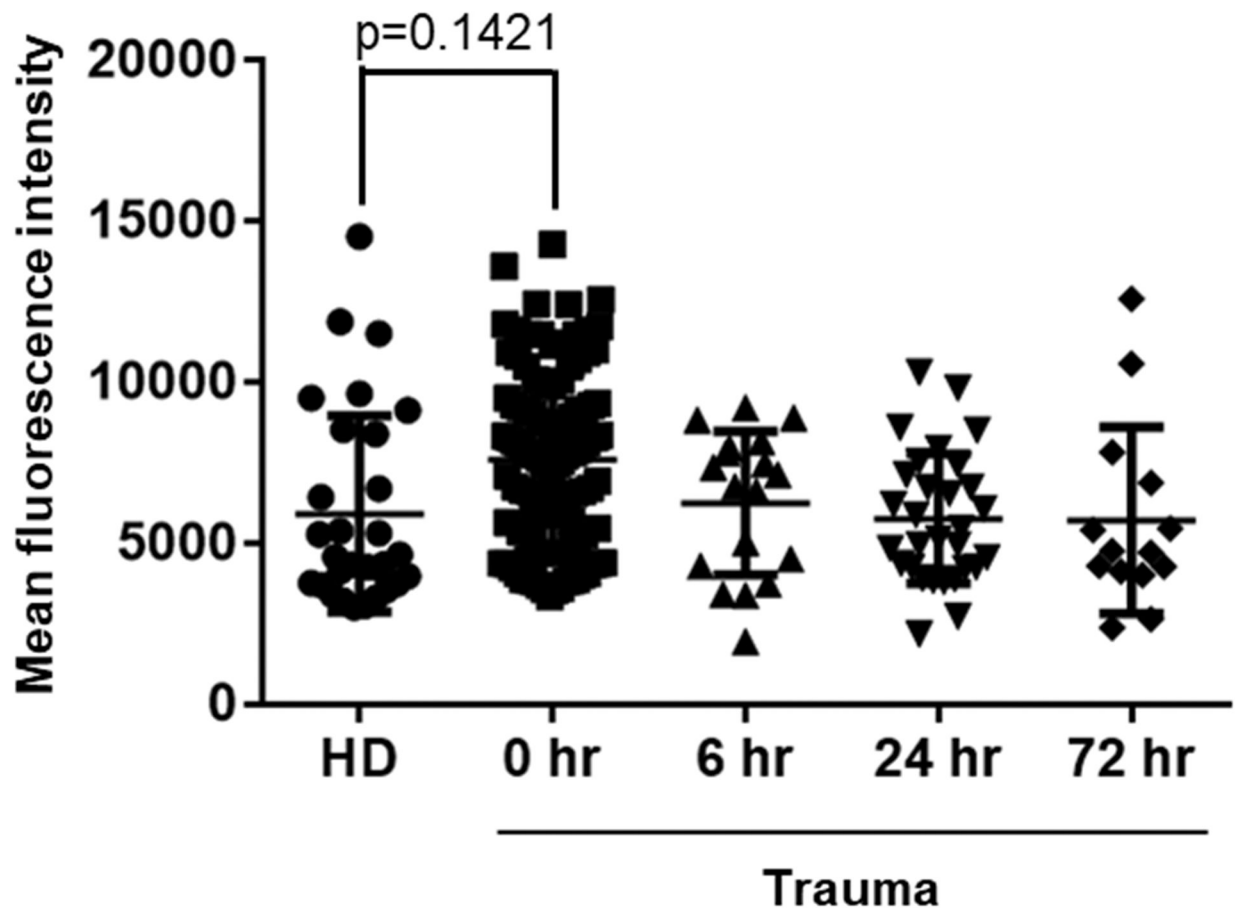


Figure 3. Increased C3d deposition on healthy RBC surfaces incubated with trauma sera. Deposition of C3d on the surface of healthy RBCs (type O, Rh negative) incubated with sera from trauma patients or healthy donors were measured by flow cytometry and mean fluorescence intensity. We used samples from 104 trauma patients (n, 0 hour =104, 6 hour =18, 24 hour =30 and 72 hour =14) and 36 samples from healthy donors (HD). The mean fluorescence intensity [Mean \pm SD (min - max)] of C3d deposition on the surface of healthy control RBCs was [5863 \pm 2868 (3025–14535)] for healthy donors, [7728 \pm 2707 (3351–14291)] for 0 hour, [6264 \pm 2234 (1959–9224)] for 6 hour, [5775 \pm 2012 (2188–10318)] for 24 hour and [5725 \pm 2897 (2384–12612)] for 72 hour patients.

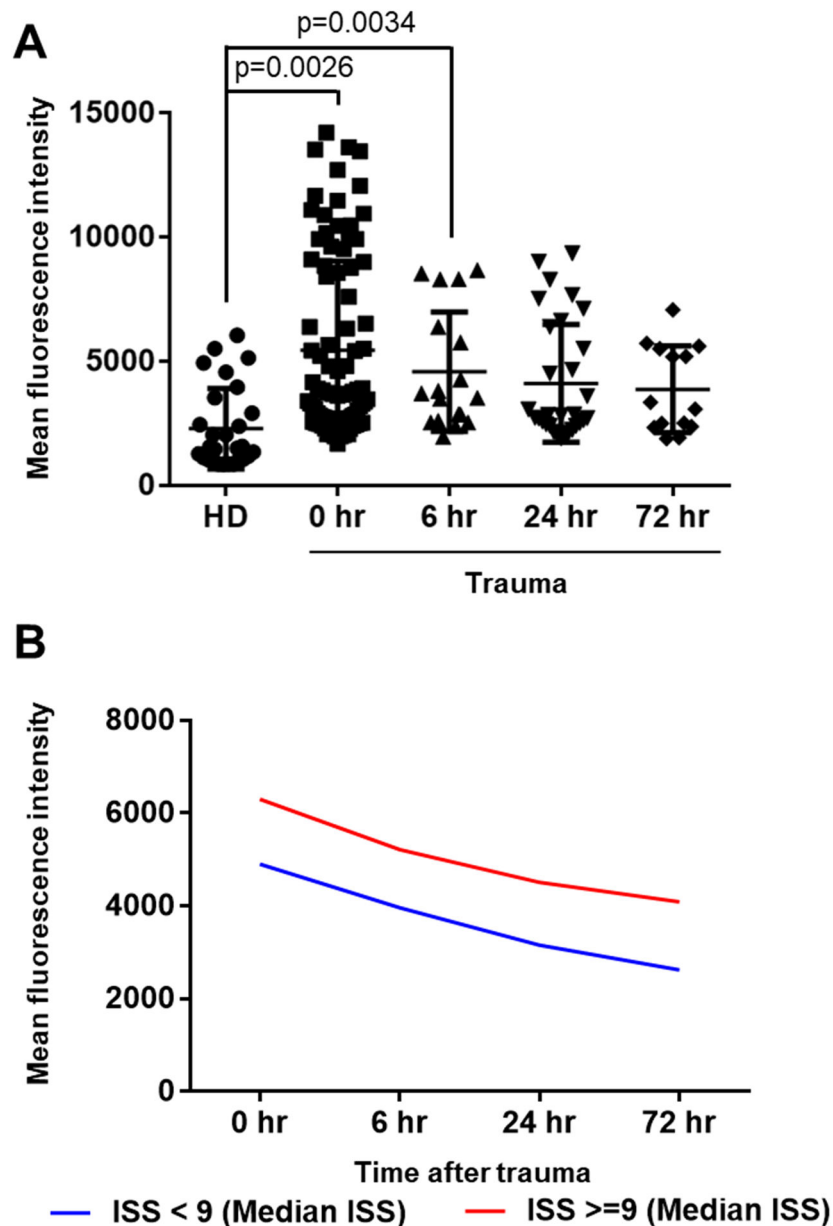


Figure 4. Increased C5b-9 deposition on healthy RBC surfaces incubated with trauma sera. **A**, the C5b9 deposition on RBC from trauma patients over time compared to normal controls by flow cytometry and mean fluorescence intensity. We used samples from 84 trauma patients (n, 0 hour =84, 6 hour =18, 24 hour =31 and 72 hour =14) and 34 samples from healthy donors (HD). The mean fluorescence intensity [Mean \pm SD (min - max)] of C5b-9 deposition on the surface of healthy control RBCs was [2331 \pm 1499 (853 –6053)] for healthy donors, [5661 \pm 3621 (1986–14217)] for 0 hour, [4590 \pm 2405 (1964–8675)] for 6 hour, [4115 \pm 2375 (1902–9352)] for 24 hour and [3883 \pm 1751 (1893– 7076)] for 72 hour patients. **B**, deposition of C5b-9 in patients with an ISS of 9 and above remains elevated up to 72 hours when compared to patients with an ISS below 9.

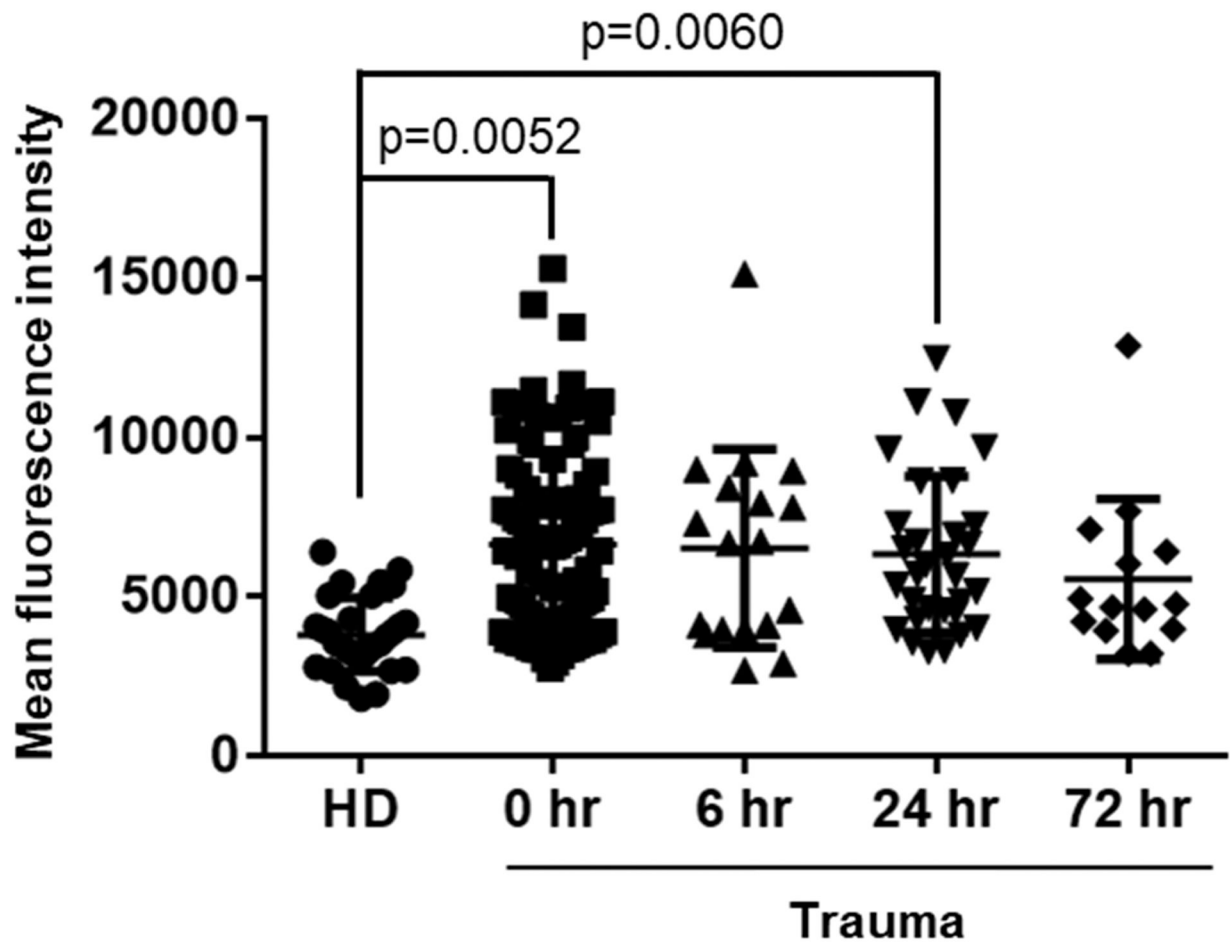


Figure 5. Phosphorylation status of band 3 in RBCs incubated with trauma sera.

Phosphorylation of band 3 in healthy RBCs (type O, Rh negative) measured by flow cytometry after incubation with sera from trauma patients or healthy donors, using eosin-5-maleimide staining. The band 3 phosphorylation in RBC from trauma patients over time compared to normal controls by flow cytometry and mean fluorescence intensity. We used samples from 97 trauma patients (n, 0 hour =97, 6 hour =18, 24 hour =31 and 72 hour =14) and 35 samples from healthy donors (HD). The mean fluorescence intensity [Mean ± SD (min - max)] of band 3 phosphorylation was [4052 ± 1380 (1806–7747)] for healthy donors, [6698 ± 2911 (2761–15314)] for 0 hour, [6522 ± 3115 (2693–15138)] for 6 hour, [6338 ± 2451 (3297–12492)] for 24 hour and [5557 ± 2517 (3217–12899)] for 72 hour patients.

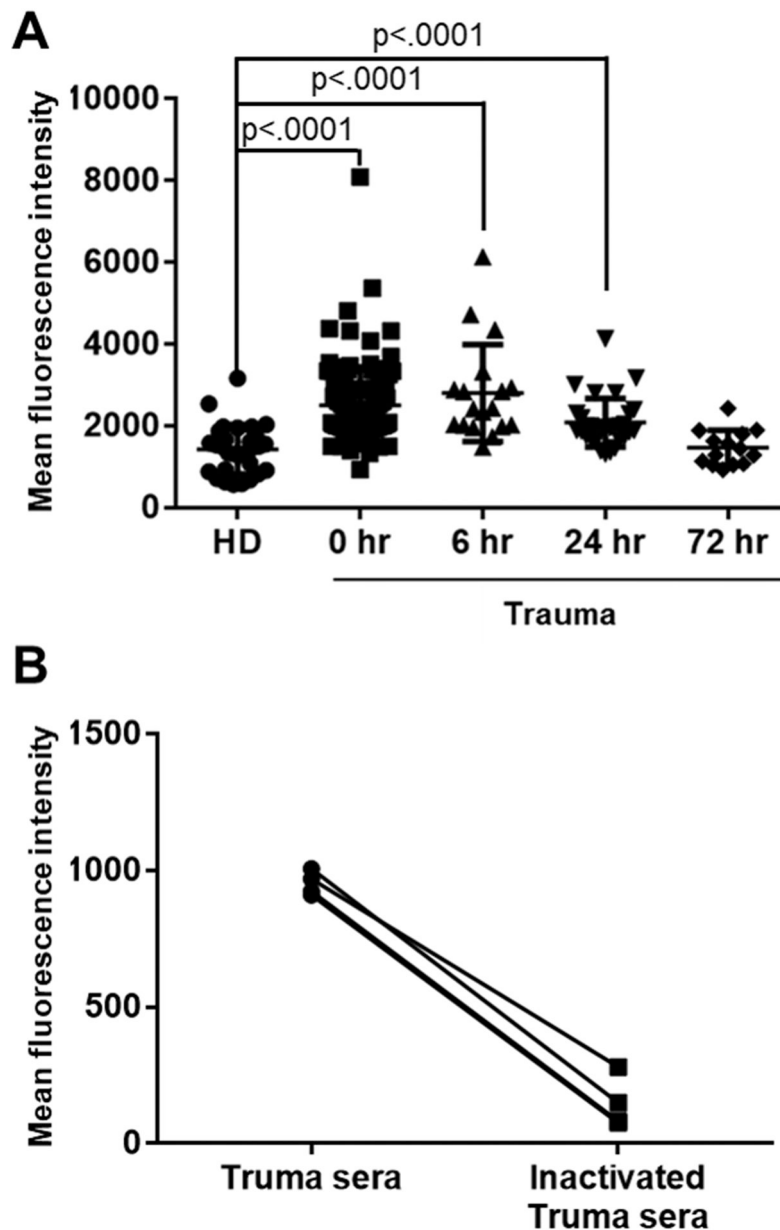


Figure 6. Nitric oxide (NO) production by RBCs induced by trauma serum.

NO production from healthy RBCs (type O, Rh negative) measured by flow cytometry after incubation with sera from trauma patients or healthy donors by using DAF-FM diacetate. NO production from RBCs was expressed as mean fluorescence intensity of DAF-FM diacetate fluorescence. **A**, we used 104 samples from trauma patients (n, 0 hour =104, 6 hour =18, 24 hour =31 and 72 hour =14) and 38 samples from healthy donors (HD). The mean fluorescence intensity [Mean \pm SD (min - max)] of nitric oxide production was [1568 \pm 730 (578 – 3455)] for healthy donors, [2542 \pm 934 (1392– 8093)] for 0 hour, [2809 \pm 1189 (1488 – 6137)] for 6 hour, [2084 \pm 594 (1318–4134)] for 24 hour and [1469 \pm 430 (935 – 2437)] for 72 hour patients. **B**, Heat inactivation of complement in sera from trauma patients eliminates their ability to trigger NO production. To inactivate complement, sera were

incubated 55°C for 30 min. RBCs were incubated with sera from trauma patients before or after inactivation of complement. Individual sera with/without inactivation of complement are shown. Straight lines are used to connect individual points to help visualize how different they are in each sample.

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Table 1.

Demographic and clinical characteristics of research subjects

Demographic Data	Healthy Donors	Trauma
Total number (n)	42	124
Age, mean (\pm SD), year	38 (4)	54 (21)
Sex, n (%)		
Male	31 (74%)	71 (57%)
Female	11 (26%)	53 (43%)
Ethnicity, n (%)*		
White	10 (37%)	75 (80%)
Black	2 (7%)	8 (9%)
Hispanic	0 (0%)	7 (7%)
Other	15 (56%)	4 (4%)

Mechanism of trauma, n (%)	
Motor vehicle crash	28 (23%)
Fall	76 (61%)
Pedestrian struck	11 (9%)
Other	10 (8%)
Organ injury	
None	73 (59%)
Brain	7 (6%)
Lung	26 (21%)
Spleen	9 (7%)
Bowel	2 (2%)
Liver	8 (6%)
Kidney	10 (8%)
Retroperitoneal Bleed	3 (2%)
Other	14 (11%)
Type of bone fracture	
None	11 (9%)
Femur	21 (17%)
Tibia or fibula	37 (30%)
Radius or ulna	23 (19%)
Humerus	11 (9%)
Hip	11 (9%)
Other	45 (36%)
Past medical history	
Alcoholism	6 (5%)
Congestive heart failure (CHF)	2 (2%)

Chronic obstructive pulmonary disease (COPD)	2 (2%)
Coronary artery disease (CAD)	5 (4%)
Chronic renal insufficiency	3 (2%)
Dementia	2 (2%)
Cerebrovascular accident (CVA)	3 (2%)
Hypertension (HTN)	32 (26%)
Intravenous drug user (IVDU)	2 (2%)
Liver disease	5 (4%)
Myocardial infarction (MI)	6 (5%)
Peptic ulcer disease (PUD)	1 (1%)
Diabetes	10 (8%)
Gout	2 (2%)
Osteoporosis	5 (4%)
Injury Severity Score, mean (\pm SD)	12.3 \pm 10.2

* Out of total 42 healthy donors, we were able to collect ethnicity information of only 27 healthy donors.

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Table 2.Mean \pm SD (min - max) of mean fluorescence intensity

	Healthy Donor	Trauma 0 hr	Trauma 6 hr	Trauma 24 hr	Trauma 72 hr
C4d RBC	2278 \pm 1504 (94–5631)	5325 \pm 2941 (1113–15683)	5254 \pm 1862 (1698–7687)	5306 \pm 1988 (1245–9462)	5074 \pm 2444 (1802–11346)
C4d	3233 \pm 1610 (1197–9215)	6071 \pm 2578 (1647–15404)	5817 \pm 2701 (2795–13764)	4753 \pm 1741 (1585–7331)	4361 \pm 2903 (2206–13450)
C3d	5863 \pm 2868 (3025–14535)	7728 \pm 2707 (3351–14291)	6264 \pm 2234 (1959–9224)	5775 \pm 2012 (2188–10318)	5725 \pm 2897 (2384–12612)
C5b-9	2331 \pm 1499 (853–6053)	5661 \pm 3621 (1986–14217)	4590 \pm 2405 (1964–8675)	4115 \pm 2375 (1902–9352)	3883 \pm 1751 (1893–7076)
Band-3	4052 \pm 1380 (1806–7747)	6698 \pm 2911 (2761–15314)	6522 \pm 3115 (2693–15138)	6338 \pm 2451 (3297–12492)	5557 \pm 2517 (3217–12899)
NO	1568 \pm 730 (578–3455)	2542 \pm 934 (1392–8093)	2809 \pm 1189 (1488–6137)	2084 \pm 594 (1318–4134)	1469 \pm 430 (935–2437)