# C4d Deposits on the Surface of Red Blood Cells in Trauma Patients and Interferes with their Function

**Takashi Muroya, MD**[1], **Lakshmi Kannan, PhD**[1], **Ionita C. Ghiran, MD**[1], **Sergey S. Shevkoplyas, PhD**[2], **Ziv Paz, MD, MPH**[1], **Maria Tsokos, MD**[1], **Jurandir J. Dalle Lucca, MD, PhD**[3], **Nathan I. Shapiro, MD MPH**[1], and **George C. Tsokos, MD**[1]

[1]Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

[2]Tulane University, New Orleans, LA

[3]Institute of Surgical Research, San Antonio, TX

## Abstract

**Objective**—Complement system is activated in patients with trauma. Although complement activation is presumed to contribute to organ damage and constitutional symptoms, little is known about the involved mechanisms. Because complement components may deposit on red blood cells (RBC), we asked whether complement deposits on the surface of RBC in trauma and whether such deposition alters RBC function.

**Design**—A prospective experimental study

**Setting**—Research laboratory

**Subjects**—Blood samples collected from 42 trauma patients and 21 healthy donors

**Intervention**—None

**Measurements and Main Results**—RBC and sera were collected from trauma patients and control donors. RBC from trauma patients (n=40) were found to display significantly higher amounts of C4d on their surface by flow cytometry compared to normal RBC (n=17) (*P*<0.01). Increased amounts of iC3b were found in trauma sera (n=27) (vs. 12 controls, *P*<0.01) by ELISA. Incubation of RBC from universal donors (O,Rh-) with trauma sera (n=10) promoted C4d deposition on their surface (vs. 6 controls, *P*<0.05). Complement-decorated RBC (n=6) displayed

limited their deformability (vs. 6 controls, *P*<0.05) in 2-dimensional microchannel arrays. Incubation of RBC with trauma sera (n=10) promoted the phosphorylation of band 3, a cytoskeletal protein important for the function of the RBC membrane (vs. 8 controls, *P*<0.05), and also accelerated calcium influx (n=9) and enhanced nitric oxide production (n=12) (vs. 4 and 8 controls respectively, *P*<0.05) in flow cytometry.

**Conclusions—**Our study found the presence of extensive complement activation in trauma patients and presents new evidence in support of the hypothesis that complement activation products deposit on the surface of RBC. Such deposition could limit RBC deformability and promote the production of nitric oxide. Our findings suggest that RBC in trauma patients malfunction, which may explain organ damage and constitutional symptoms that is not accounted for otherwise by previously known pathophysiologic mechanisms.

## Keywords

red blood cell; trauma; C4d; complement; deformability; nitric oxide

## Introduction

The clinical significance of trauma cannot be underestimated with over 10 million car accidents occurring and over 35 thousand people dying each year in the U.S. (1). Trauma resulting from accidents or unintentional injuries is the first leading cause of death for those under 50 years old and accounts for one out of about every 20 deaths in the U.S. (2).

Although trauma usually involves a certain anatomical injury involving extremities or the torso and is followed by obvious pathophysiological events usually linked to blood loss, it is invariably associated with manifestations from organs not directly affected by injury. The origin of these secondary manifestations is poorly understood. There is information suggesting the presence of an acute inflammatory response in patients experiencing trauma. Specifically, there is evidence that complement system is activated and mediates early post-traumatic inflammatory response (3-5). In addition, the levels of proinflammatory cytokines, including TNF-á, IL-1 and IL-6 are increased in trauma (6, 7). It is suggested that complement activation and proinflammatory cytokine storm occurring in patients with trauma collectively choreograph systemic inflammatory response syndrome associated with acute respiratory distress syndrome and multiple organ failure (8).

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), which limits their deformability while promoting nitric oxide (NO) production (9). It has been reported that RBC from patients suffering from hemorrhagic shock displays reduced deformability, show altered morphological appearance, and contribute to the reduction of blood flow to distant organs (10, 11).

Based on the evolving studies, we hypothesized that complement activation affects RBC function in trauma patients. In this communication we demonstrate that complement becomes activated in trauma patients and split products are deposited on the surface of RBC limiting their ability to pass through capillary-size microchannels and increasing their

capacity to produce NO. Our findings suggest that the inhibition of complement can serve as an adjunct treatment in trauma patients.

## Materials and Methods

### Participants

This prospective experimental study was approved by the institutional Review Board of Beth Israel Deaconess Medical Center, and was carried out as anonymized wasted samples under a waiver of consent. Total of 42 patients who visited the emergency room at Beth Israel Deaconess Medical Center for trauma from July 2012 to March 2013 were enrolled nonconsecutively. Trauma patients were 18 years and older, and have been admitted for bone fracture, organ injury or head trauma. They received a blood test as required for their care. Patients carrying the diagnosis of cancer, infection, or autoimmune diseases were excluded. None of the patients had received a transfusion prior to sampling. Healthy donors (n=21) were recruited from the Division of Rheumatology at Beth Israel Deaconess Medical Center after written informed consent. They did not have an apparent illness. RBC were collected from 40 out of 42 trauma patients (2 samples were destroyed during the sample collection process) and 17 healthy donors and sera were collected from 27 patients and 13 healthy donors. Patients' clinical data were recorded including age, sex, ethnicity, mechanism of injury, and existing injury. Injury Severity Score (ISS) was also calculated.

### Antibodies and reagents

Primary antibodies were anti-C4d monoclonal antibody (A213; Quidel, San Diego, CA) and IgG1k-isotype control (401402; BioLegend, San Diego, CA). Secondary antibody was Alexa Fluor 488-conjugated rabbit anti-mouse IgG (A11059; Life technologies, Grand Island, NY). Other reagents were as follows: Hanks' balanced salt solution with $Ca^{2+}$ and $Mg^{2+}$ ($HBSS^{++}$) (14025; Life technologies), IgG-free bovine serum albumin (BSA) (A9085; Sigma-Aldrich, St Louis, MO), DAF-FM diacetate (D23844; Life technologies), Flio-4 AM (F14217; Life technologies), and eosin-5-maleomide (E118; Life technologies).

### Measurement of serum iC3b levels

The concentration of iC3b present in serum was measured using the MicroVue iC3b Enzyme Immunoassay kit (A006; Quidel)

### RBC preparation and incubation with sera

Heparinized blood or serum of trauma patients was obtained from the Emergency Department after clinical examination, and control blood or serum was obtained from healthy adult volunteers. The sample collection was performed in accordance with guidelines of the Institutional Review Board of Beth Israel Deaconess Medical Center after informed consent in accordance with the Declaration of Helsinki. Blood was collected in heparin-lithium tubes for RBC and serum in serum-separator tubes. The serum was stored at −80°C. Heparinized blood was washed in $HBSS^{++}$ before use.

For serum studies, on the day of the experiment, the serum samples were thawed from various trauma patients and healthy donors and were used for different measurements under

the same conditions. Blood from healthy universal donors (type O, Rh negative) was obtained in heparin-lithium tube 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 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 7.6.5 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.

### Analysis of intracellular calcium influx

RBC from healthy universal donors were preloaded with Fluo-4 AM for 15 minutes at 37°C, then, washed to remove any dye that is nonspecifically bound with RBC surface, and resuspended in HBSS$^{++}$. Loaded RBC were incubated at room temperature for an additional 10 minutes to allow complete de-esterification of intracellular AM esters. Fluorescence levels of RBC were acquired by FACScan for establishing the baseline of intracellular $Ca^{2+}$ concentration. After 60 seconds, one hundred microliters of control or trauma serum was quickly added to the RBC (20% of final concentration), and the change of the fluorescence intensity that indicates intracellular $Ca^{2+}$ concentration was recorded for an additional 3 minutes.

### Measurement of NO production

RBC from healthy universal donors preloaded with DAF-FM diacetate in BSA-free HBSS$^{++}$ for 30 minutes at 37°C, 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.

### Measurement of RBC deformability using 2-dimensional (2-D) filters

To measure the ability of RBC to undergo capillary-like deformations, we used a 2-D microchannel array as previously described (12) that uses the hydrostatic pressure to drive the RBC through the microchannel array. For each experiment, the device was calibrated using as reference the zero pressure difference corresponded to the absence of movement of RBC within the device. RBC (8 μl, hematocrit 20%) were loaded into the inlet reservoir and

driven into the capillary-like area by lowering the waste reservoir tubing. Once RBC entered the capillary-like area, the waste reservoir tubing was raised at a height that allowed RBC to pass through the 25-μm length of the capillary in about 3 seconds. The cells were recorded using a 40x0.75 Ph2 Plan Fluorite objective on a TE300 Nikon inverted microscope (Nikon Inc., Melville, NY), using a Retiga Exi (QImaging, Surrey, Canada) CCD camera controlled with iVision 4.7 software (BioVison, Exton, PA) at a rate of 10 frames/second. Movies representing 600 frames then were analyzed off-line frame by frame, and the results were expressed as the number of seconds from entry to egress (9). RBC that displayed unusual shapes (e.g. echinocytes, acanthocytes) were excluded from the measurement.

### Statistical analysis

SPSS version 17.0 for Windows (SPSS, Chicago, IL) was used for all statistical analysis. Mann-Whitney U test was used for between group analysis and values were showed as medians [interquartile range] unless otherwise stated. Correlation between the ISS and the amount of C4d deposition on trauma RBC was assessed by Spearman correlation coefficient. Paired t-test analysis was performed wherever necessary. A value of $P < 0.05$ was considered statistically significant.

## Results

### Patient characteristics

A total of 42 patients were included in this study (Table 1). The patient group consisted of 33 men, and 9 women, with mean age of 47 years. Most of the patients presented with blunt injury resulting from motor vehicle crash (n=19) or fall (n=16). The median value of ISS was 17 [9-25].

### Deposition of complement activated product C4d on RBC from trauma patients

To determine the extent of complement deposition on the surface of RBC of trauma patients, we used 40 samples from trauma patients and 17 samples from healthy donors. RBC from trauma patients displayed significant C4d deposition on their membrane as compared to RBC from normal healthy donor (565 [443-886] versus 386 [309-489], $P < 0.01$, Table 2). A representative tracing is shown in Figure 1A and cumulative data in Figure 1B. We did not find a correlation between the ISS and the amount of C4d deposited on the surface of RBC (data not shown).

It has been reported previously that complement becomes activated in patients who suffer various forms of trauma (4, 13). To confirm that complement activation occurred in our trauma patients we measured the iC3b levels in the sera obtained from trauma patients and healthy donors. Indeed, iC3b levels were significantly higher in the sera of trauma patients (n=27) compared to sera from healthy donors (n=12) (10.7 [8.2-14.0] *versus* 6.6 [5.3-9.4] $P < 0.01$) (Figure 1C).

### C4d deposition on healthy RBC incubated with sera from trauma patients

In order to evaluate the effect of complement deposition on various aspects of RBC function, we first established a method to show that incubation of normal RBC from

universal donors (type O, Rh negative) with sera from trauma patients results in complement deposition on the surface of RBC. Indeed, as shown in Figure 2A (representative experiment) and Figure 2B (cumulative data), incubation of normal RBC with sera from trauma patients (n=10) resulted in a significant C4d deposition on the membrane of RBC as compared to sera from healthy controls (n=6) (289 [282-300] versus 267 [261-272], $P<0.05$). Removal of $Ca^{2+}$ by adding 10mM EDTA to the serum decreased the deposition of C4d on the surface of RBC (Figure 2C), suggesting that activation of complement through the classical pathway accounts for the deposition of complement on RBC (14).

### Decreased RBC membrane deformability by trauma sera

It has been previously reported that complement deposition on RBC can render their membrane less deformable, limiting the ability of RBC to pass through capillaries (15). We evaluated RBC membrane deformability by using a microfluidic device as described in the Materials and Methods section (Figure 3A) and measured the time it took RBC to pass through the microchannels. RBC from a universal donor were treated either with sera from normal individuals (n=6) or trauma patients (n=6). Individual dot blots are presented in Figure 3B and cumulative data in Figure 3C. Our results show that exposure of RBC to sera from trauma patients limits their ability to pass through capillary-size microchannels (2.03 [1.96-2.25] versus 1.85 [1.81-1.90], $P<0.05$).

### Phosphorylation status of band 3

RBC membrane deformability is related to the phosphorylation status of membrane proteins such as β-spectrin or band 3 (16, 17). To determine whether band 3 becomes phosphorylated in RBC exposed to trauma sera, we treated RBC exposed to control (n=8) or trauma sera (n=10) 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 levels (17). Binding of eosin-5-maleimide to RBC has been used previously to estimate the phosphorylation levels of band 3 (9, 17), and here we similarly used eosin-5-maleimide to estimate the tyrosine phosphorylation levels of band 3. As shown in Figure 4, band 3 of RBC was phosphorylated after incubation with trauma sera compared to incubation with normal sera (89.3 [88.3-92.3] versus 84.8 [83.0-85.5], $P<0.05$).

### Promotion of $Ca^{2+}$ influx

Tyrosine phosphorylation level of band 3 is known to correlate with intracellular $Ca^{2+}$ concentration (18, 19). We asked whether exposure of RBC to trauma sera results in increased $Ca^{2+}$ entry. As shown in Figure 5A (representative tracings) and Figure 5B (cumulative data), the addition of trauma sera (n=9) to Fluo-4 AM- loaded RBCs resulted in increased $Ca^{2+}$ concentrations into the RBC compared to RBC to which normal sera (n=4) were added (1.08 [1.05-1.12] versus 1.03 [1.02-1.04], $P<0.05$).

### Induction of the NO production

Recently, it was reported that human RBC express an active and functional endothelial-type NO synthase (eNOS) which is present in the membrane and in the cytoplasm (20). In RBC, mechanical stress (shear stress) stimulates NO generating mechanisms and $Ca^{2+}$ is important

in this process (21). Having observed that exposure of RBC to trauma serum results in increased intracellular $Ca^{2+}$ concentrations, we asked whether it also results in an increased NO production. Our results showed that exposure of RBC to trauma sera (n=12) promoted NO production compared to exposure to normal sera (n=8) (543 [527-576] versus 453 [425-501], *P*<0.05) (Figure 6A, representative tracing, and Figure 6B, cumulative data). Increased NO production results from the deposition of complement on the surface of RBC because heat inactivation of sera (55°C for 30 minutes) eliminated the ability of trauma sera to increase NO production by RBC (Figure 6C) (597±59 versus 521±21, mean±SD, paired t-test, *P*<0.05).

## Discussion

Complement becomes activated in patients suffering various types of trauma and this was confirmed in our patients by demonstrating increased levels of iC3b in their sera. Uncontrolled complement activation, common in a wide-variety of conditions both chronic and acute, has multiple deleterious effects on various circulating blood cells as well as on organ function primarily through the action of inflammatory split products (anaphylatoxins) such as C3a and C5a (22, 23). To date, there is little data regarding the effects of the non-inflammatory complement by-products, mainly C3b and C4b generated during complement activation on circulating cell function, specifically RBC. In this study we show that C4d, a split product of C4, deposits on the surface of RBC, thus limiting their ability to pass through narrow microchannels and enhancing 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 that mediates phosphorylation of band 3.

C4d is the stable remnant of C4 and it has been shown to remain on the surface of RBC in patients with SLE for a prolonged period of time (24, 25). In our study, we demonstrated increased deposition of C4d on the surface of RBC from patients with various degrees and types of trauma; yet, we failed to demonstrate a direct correlation between ISS determined clinically and levels of C4d deposition on the surface of RBC. One possible explanation we were not able to establish the correlation is the logistics involved in handling trauma patients and the collection of blood or sera samples. The time that lapsed between trauma and the collection of samples could not be controlled, which could have affected the overall amounts of C4d that had accumulated on the surface of RBC.

Following the two key observations- the increased complement activation in sera and increased deposition of complement fragment C4d on RBC in trauma patients- we chose to test whether the increase in complement activation and deposition leads to RBC dysfunction. Should binding of complement on the surface of RBC have a functional effect, it should be occurring at early time points. To explore this, RBC from universal donors were incubated with sera from trauma patients who showed enhanced deposition of C4d on RBC. As noted, exposure of RBC to trauma serum resulted in limitation of their ability to pass through capillaries, increased phosphorylation of band 3, $Ca^{2+}$ entry, and NO production. The alterations in RBC function in trauma patients could be due to deposition of complement fragments on the RBC membranes as others and we have shown both *in vitro* and *ex vivo* using RBC from normal individuals (15) and SLE patients (9).

The functional consequences of the deposition of complement on the surface of RBC cannot be overstated. In order for RBC to perform their main function, that is to deliver oxygen to tissues, they must pass through narrower (<8 μm in diameter) capillaries and to do that they have to be able to deform (26, 27). If deposition of C4d renders them difficult to deform and reach tissues, then hypoxia will ensue. 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 (28). In addition, NO derived from eNOS present in RBC also directly regulates and maintains RBC deformability (20, 29). In our study we demonstrated that under trauma conditions, increased complement deposition decreases RBC deformability and increases NO production. According to the existing literature, under normal conditions, increase in NO production improves microvascular blood flow. However in our studies, although the NO production is high in trauma patients, we observe a decrease in RBC deformability. We speculate that this may be due to C4d deposition on RBC in trauma patients that may have a prominent effect on RBC deformability than compared to NO effect on RBC deformability. The increased production of NO by C4d-decorated RBC in the microcirculation may further alter epithelial and smooth muscle cell function and influence the clinical picture in trauma patients.

Our studies have certain limitations including the fact that the studied cohort was quite diverse. 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. Further studies need to be done in clinically homogenous trauma patients compared to matched controls. The second limitation is that although we made every effort to minimize the time between the collection of the blood sample and the performance of the experiments, we could not control the time between the occurrence of traumatic injury and the time the patient was admitted to the emergency room. As indicated above, during that time, variable amounts of C4d could have been deposited on the surface of RBC. Yet, the studies in which RBC from universal donors were exposed to trauma serum collected and stored properly lend validity to our conclusions. A proper prospective study (e.g. collection of serial blood samples from trauma patients during the first hours or days after admission) should be more informative. Third, for the serum-based experiments, instead of whole study sample, we used sera from trauma patients who showed increased C4d deposition on RBC. In the current study, our goal was to narrow down our experimental design from an epidemiologic standpoint to a proof-of concept study to further understand the molecular mechanisms.

## Conclusions

We conclude 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. Thus it may contribute to trauma-associated morbidity and mortality. As a next step, we plan to conduct a prospective study with larger population, which includes clinically homogenous trauma patients compared to matched controls. We believe that our present and future

findings might suggest modalities that limit complement activation in trauma patients to be considered for clinical trials.

## Acknowledgments

## References

1. U.S. Census Bureau. Statistical Abstract of the United States: 2012. 131th edition. U.S. Department of Commerce; Washington, DC: 2011.

2. Hoyert DL, Xu J. Deaths: preliminary data for 2011. Natl Vital Stat Rep 2012. 61(6):1–51.

3. Neher MD, Weckbach S, Flierl MA, et al. Molecular mechanisms of inflammation and tissue injury after major trauma--is complement the "bad guy"? J Biomed Sci. 2011; 18:90. [PubMed: 22129197]

4. Fosse E, Pillgram-Larsen J, Svennevig JL, et al. Complement activation in injured patients occurs immediately and is dependent on the severity of the trauma. Injury. 1998; 29(7):509–514. [PubMed: 10193492]

5. Szebeni J, Baranyi L, Savay S, et al. Complement activation during hemorrhagic shock and resuscitation in swine. Shock. 2003; 20(4):347–355. [PubMed: 14501949]

6. Marik PE, Flemmer M. The immune response to surgery and trauma: Implications for treatment. J Trauma Acute Care Surg. 2012; 73(4):801–808. [PubMed: 22976420]

7. Hirsiger S, Simmen HP, Werner CM, et al. Danger signals activating the immune response after trauma. Mediators Inflamm. 2012; 2012:315941. [PubMed: 22778496]

8. Rittirsch D, Redl H, Huber-Lang M. Role of complement in multiorgan failure. Clin Dev Immunol. 2012; 2012:962927. [PubMed: 23320021]

9. Ghiran IC, Zeidel ML, Shevkoplyas SS, et al. Systemic lupus erythematosus serum deposits C4d on red blood cells, decreases red blood cell membrane deformability, and promotes nitric oxide production. Arthritis Rheum. 2011; 63(2):503–512. [PubMed: 21280005]

10. Machiedo GW, Zaets SB, Berezina TL, et al. Trauma-hemorrhagic shock-induced red blood cell damage leads to decreased microcirculatory blood flow. Crit Care Med. 2009; 37(3):1000–1010. [PubMed: 19237910]

11. Berezina TL, Zaets SB, Machiedo GW. Alterations of red blood cell shape in patients with severe trauma. J Trauma. 2004; 57(1):82–87. [PubMed: 15284553]

12. Glodek AM, Mirchev R, Golan DE, et al. Ligation of complement receptor 1 increases erythrocyte membrane deformability. Blood. 2010; 116(26):6063–6071. [PubMed: 20861458]

13. Ganter MT, Brohi K, Cohen MJ, et al. Role of the alternative pathway in the early complement activation following major trauma. Shock. 2007; 28(1):29–34. [PubMed: 17510601]

14. Major B, Kardos J, Kekesi KA, et al. Calcium-dependent conformational flexibility of a CUB domain controls activation of the complement serine protease C1r. J Biol Chem. 2010; 285(16): 11863–11869. [PubMed: 20178990]

15. Karnchanaphanurach P, Mirchev R, Ghiran I, et al. C3b deposition on human erythrocytes induces the formation of a membrane skeleton-linked protein complex. J Clin Invest. 2009; 119(4):788–801. [PubMed: 19258706]

16. Manno S, Takakuwa Y, Nagao K, et al. Modulation of erythrocyte membrane mechanical function by beta-spectrin phosphorylation and dephosphorylation. J Biol Chem. 1995; 270(10):5659–5665. [PubMed: 7890688]

17. Condon MR, Feketova E, Machiedo GW, et al. Augmented erythrocyte band-3 phosphorylation in septic mice. Biochim Biophys Acta. 2007; 1772(5):580–586. [PubMed: 17382523]

18. Minetti G, Piccinini G, Balduini C, et al. Tyrosine phosphorylation of band 3 protein in Ca2+/A23187-treated human erythrocytes. Biochem J. 1996; 320(Pt 2):445–450. [PubMed: 8973551]

19. Zipser Y, Piade A, Barbul A, et al. Ca2+ promotes erythrocyte band 3 tyrosine phosphorylation via dissociation of phosphotyrosine phosphatase from band 3. Biochem J. 2002; 368(Pt 1):137–144. [PubMed: 12175337]

20. Kleinbongard P, Schulz R, Rassaf T, et al. Red blood cells express a functional endothelial nitric oxide synthase. Blood. 2006; 107(7):2943–2951. [PubMed: 16368881]

21. Ulker P, Meiselman HJ, Baskurt OK. Nitric oxide generation in red blood cells induced by mechanical stress. Clin Hemorheol Microcirc. 2010; 45(2-4):169–175. [PubMed: 20675897]

22. Baldwin WM 3rd, Kasper EK, Zachary AA, et al. Beyond C4d: other complement-related diagnostic approaches to antibody-mediated rejection. Am J Transplant. 2004; 4(3):311–318. [PubMed: 14961982]

23. Fleming SD, Phillips LM, Lambris JD, et al. Complement component C5a mediates hemorrhage-induced intestinal damage. J Surg Res. 2008; 150(2):196–203. [PubMed: 18639891]

24. Manzi S, Navratil JS, Ruffing MJ, et al. Measurement of erythrocyte C4d and complement receptor 1 in systemic lupus erythematosus. Arthritis Rheum. 2004; 50(11):3596–3604. [PubMed: 15529364]

25. Kao AH, Navratil JS, Ruffing MJ, et al. Erythrocyte C3d and C4d for monitoring disease activity in systemic lupus erythematosus. Arthritis Rheum. 2010; 62(3):837–844. [PubMed: 20187154]

26. Diez-Silva M, Dao M, Han J, et al. Shape and Biomechanical Characteristics of Human Red Blood Cells in Health and Disease. MRS Bull. 2010; 35(5):382–388. [PubMed: 21151848]

27. Mohandas N, Gallagher PG. Red cell membrane: past, present, and future. Blood. 2008; 112(10): 3939–3948. [PubMed: 18988878]

28. Baskurt OK, Ulker P, Meiselman HJ. Nitric oxide, erythrocytes and exercise. Clin Hemorheol Microcirc. 2011; 49(1-4):175–181. [PubMed: 22214688]

29. Bor-Kucukatay M, Wenby RB, Meiselman HJ, et al. Effects of nitric oxide on red blood cell deformability. Am J Physiol Heart Circ Physiol. 2003; 284(5):H1577–1584. [PubMed: 12521942]
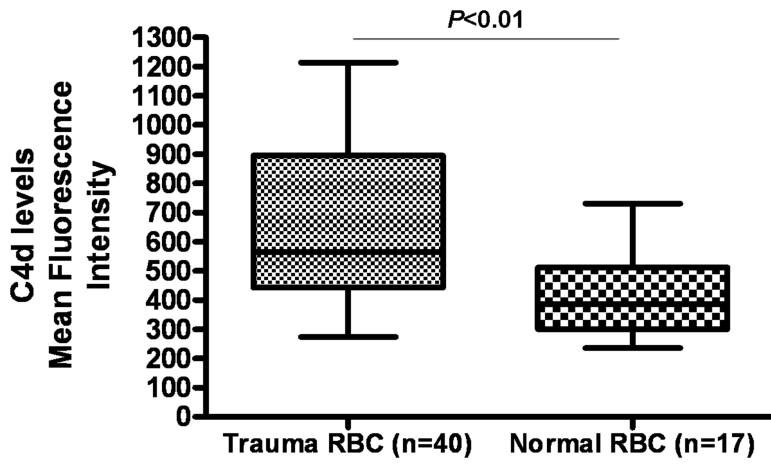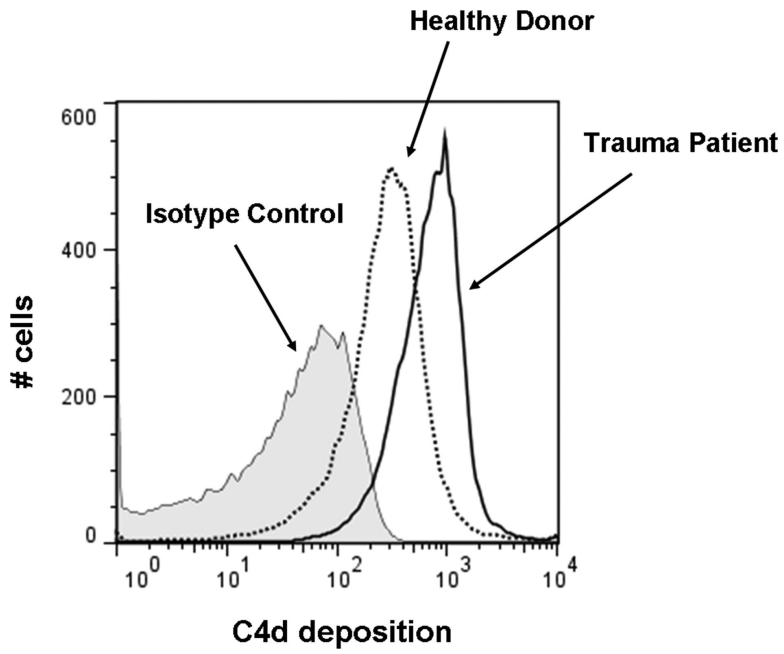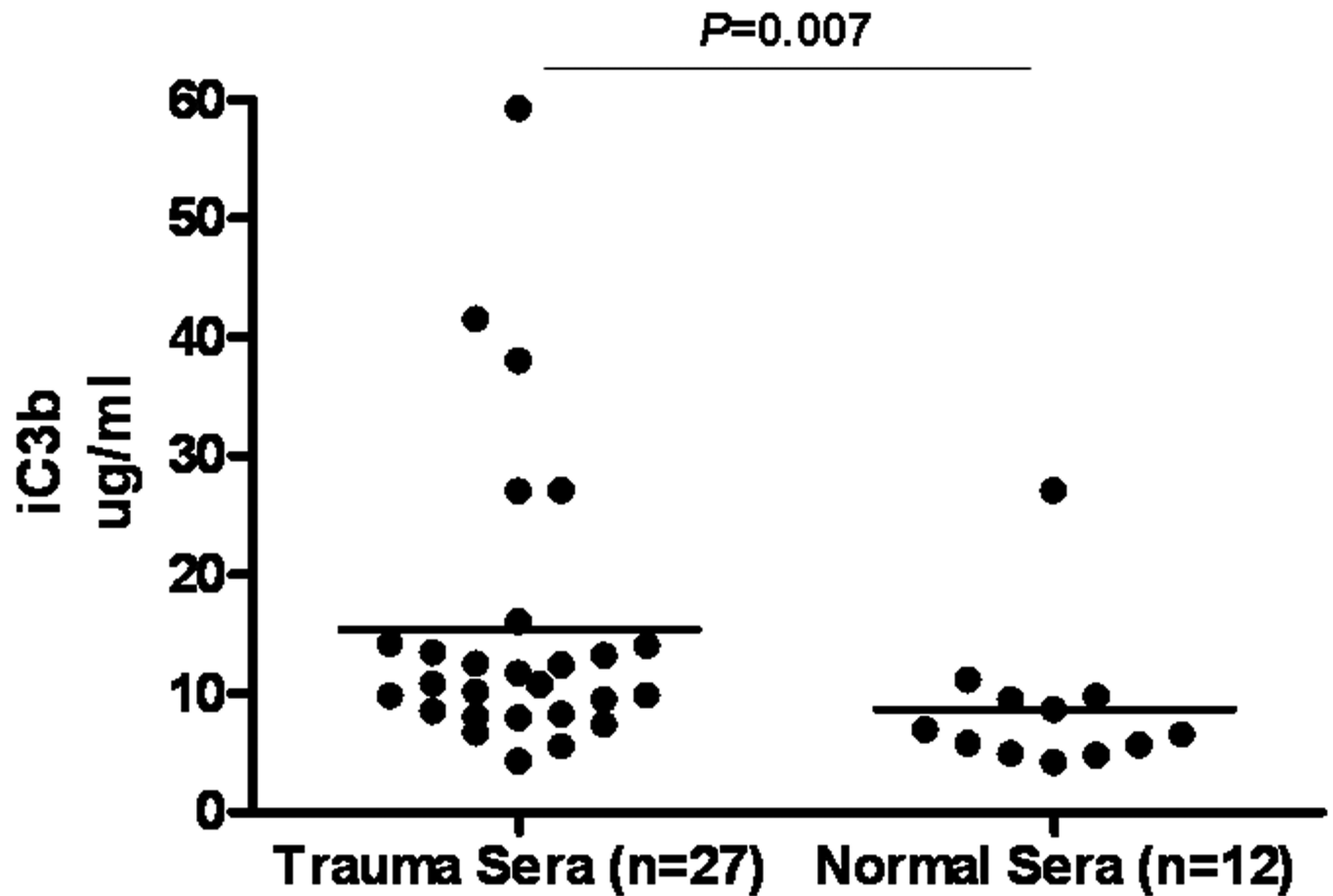
**Figure 1. Increased C4d deposition on the surface of membrane of red blood cells from trauma**
**A**, Deposition of C4d on the surface of red blood cells from trauma patients and healthy donors was measured by flow cytometry. Representative of experiments is shown.
**B**, Data of C4d deposition on red blood cells from all samples was expressed as mean fluorescence intensity and cumulative data from trauma patients (n=40) or normal healthy donors (n=17) are shown as box plots. Each box shows the 25th and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median.
**C**. Serum levels of iC3b fragments from trauma patients (n=27) and from normal healthy donors (n=12) were assayed as a measure of activation of complement. Horizontal lines indicate the median.
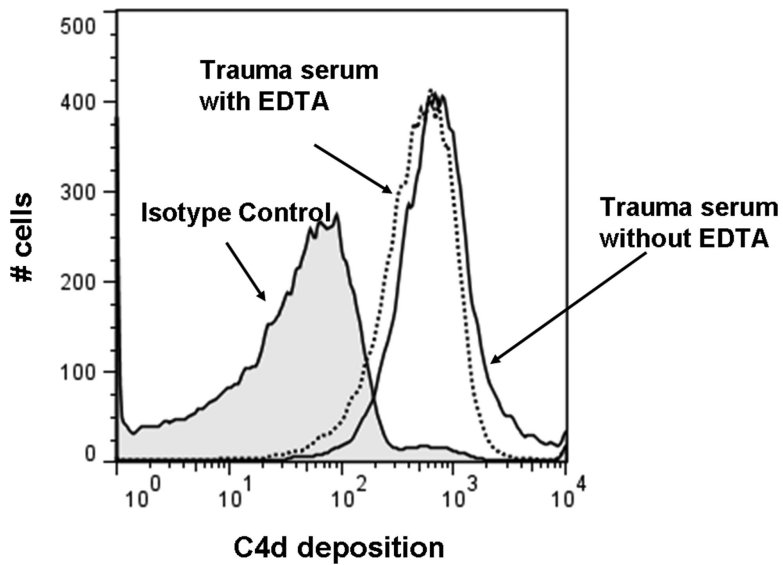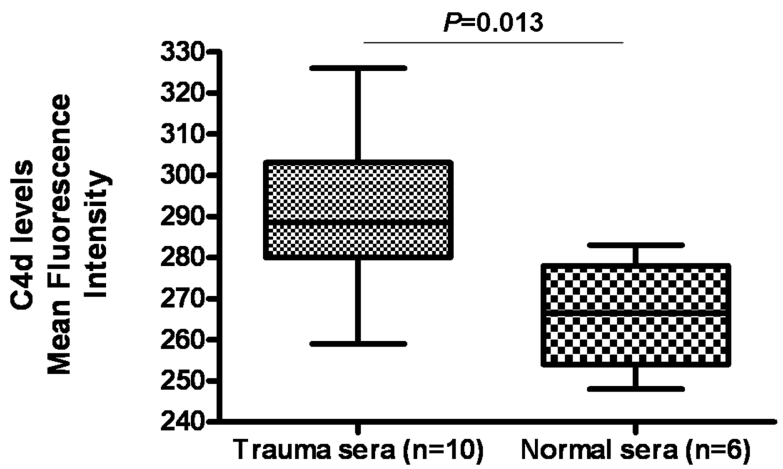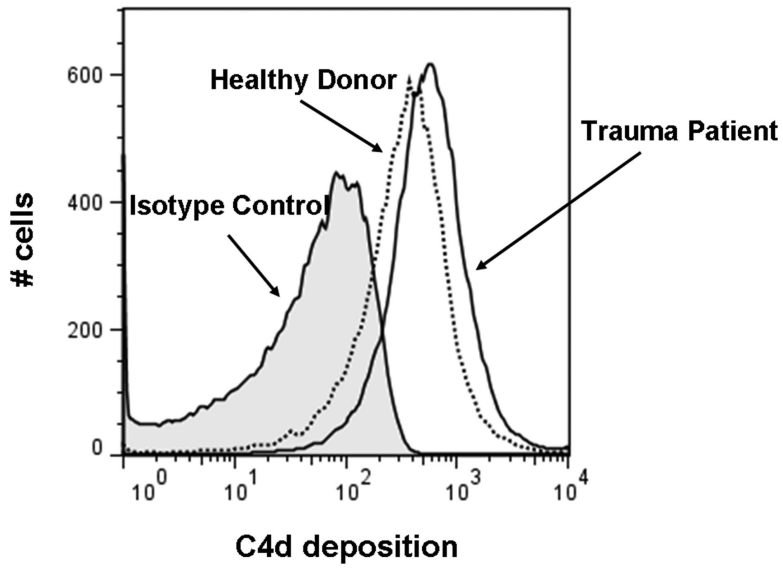
**Figure 2. Increased C4d deposition on healthy red blood cell membranes incubated with trauma sera**

**A**. Deposition of C4d on the surface of healthy red blood cells (type O, Rh negative) incubated with sera from trauma patients or healthy donors were measured by flow cytometry. Representative data is shown.

**B**. Data of C4d deposition on red blood cells from all samples was expressed as mean fluorescence intensity, and cumulative data from trauma patients (n=10) or healthy donors (n=6) are shown as box plots. Each box shows the 25th and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median.

**C**. Deposition of C4d on the surface of healthy red blood cells (type O, Rh negative) incubated with sera from trauma patients with or without 10 mM EDTA. Representative data is shown.
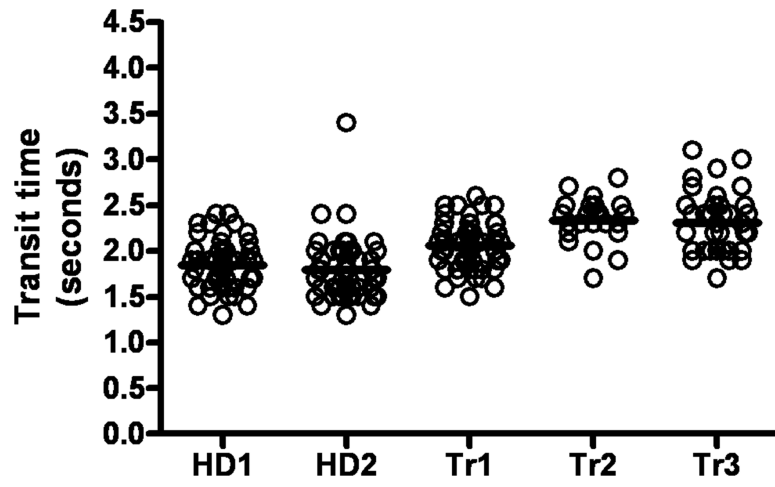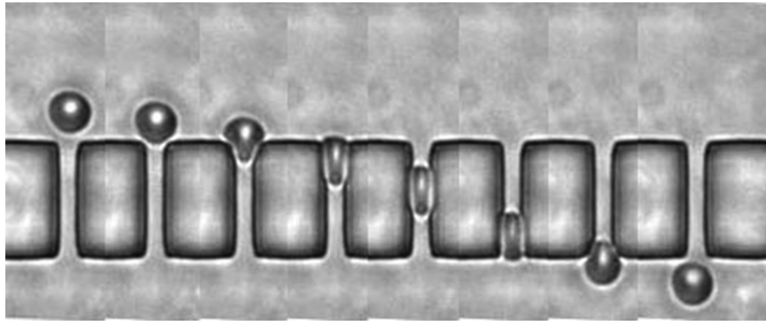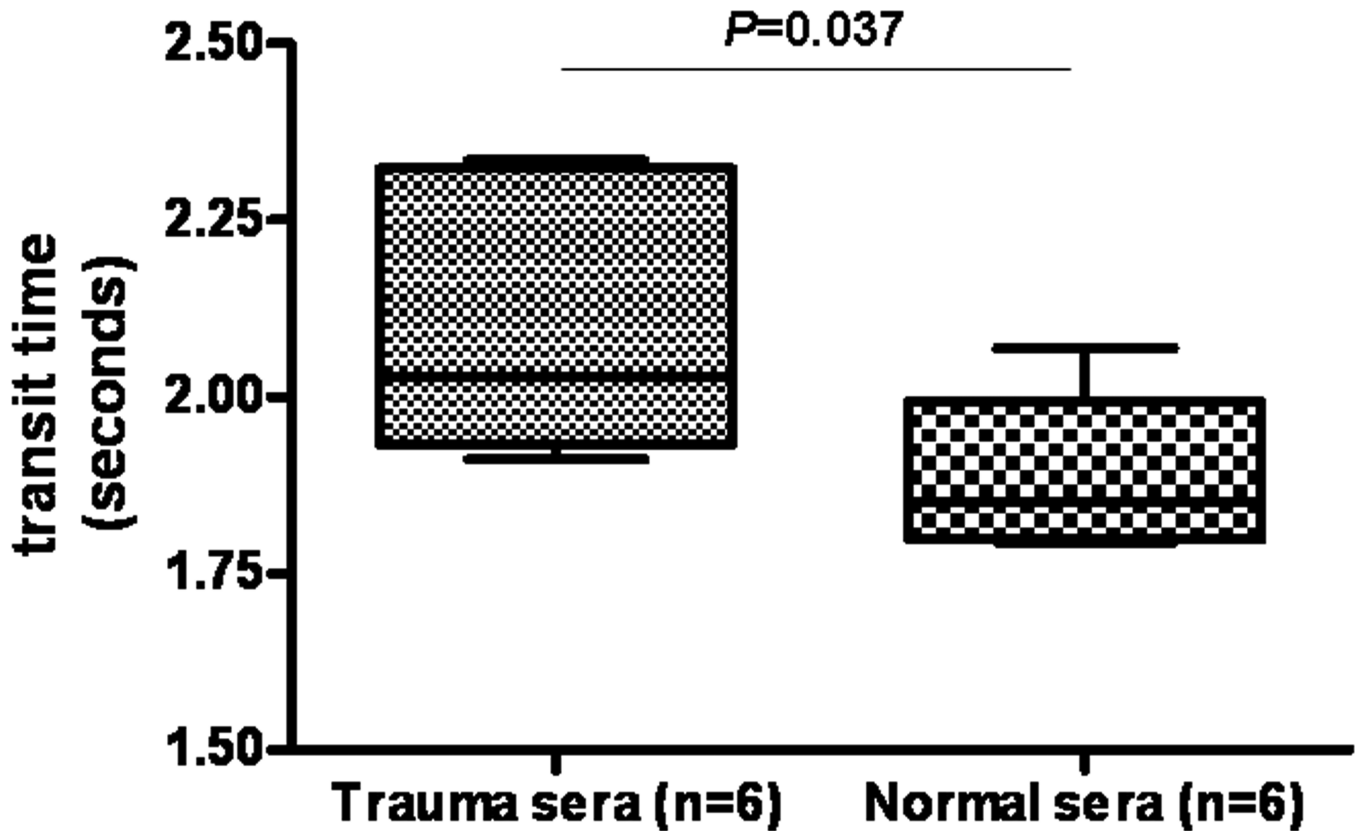
**Figure 3. Decreased red blood cell membrane deformability after incubation with trauma sera**
**A.** A composite series of images illustrating the passage of a single red blood cell through a capillary-size microchannel of the 2- dimensional filter device
**B.** Healthy universal donor's (type O, Rh negative) red blood cells deformability were measured by using the 2-dimensional filter device after incubation with sera from trauma patients or healthy donors. Decreased deformability of each red blood cell was associated with increased transit time (i.e. the time it took the cell to pass through the capillary microchannels of 2-dimensional filter). Data are shown as dot blots of all experiments with each circle showing the passage time for one red blood cell. Horizontal lines show the mean. Representative data are shown.
**C.** Cumulative data from trauma patients (n=6) or healthy donors (n=6) are shown. Data are expressed as box plots. Each box shows the 25th and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median.
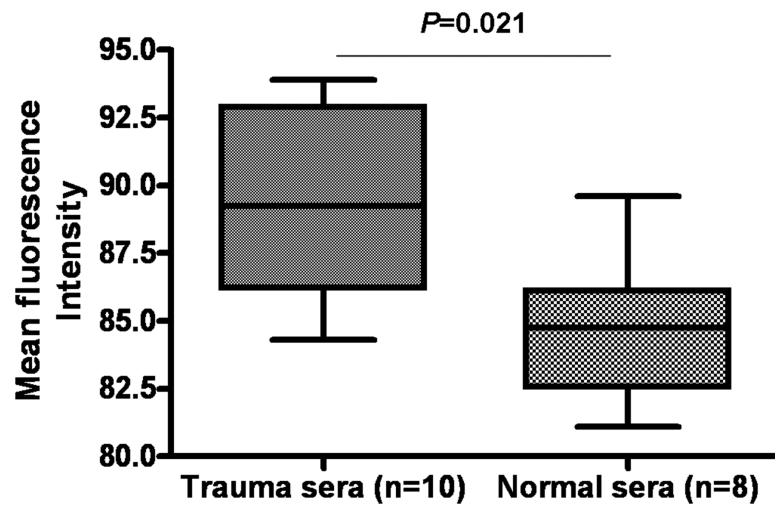
**Figure 4. Phosphorylation status of band 3 in red blood cells incubated with trauma sera**
Phosphorylation of band 3 in healthy red blood cells (type O, Rh negative) measured by flow cytometry after incubation with sera from trauma patients or healthy donors, using eosin-5-maleimide staining. Band 3 phosphorylation was expressed as mean fluorescence intensity of eosin-5-maleimide fluorescence, and cumulative data from trauma patients (n=10) or healthy donors (n=8) are shown as box plots. Each box shows the 25th and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median.
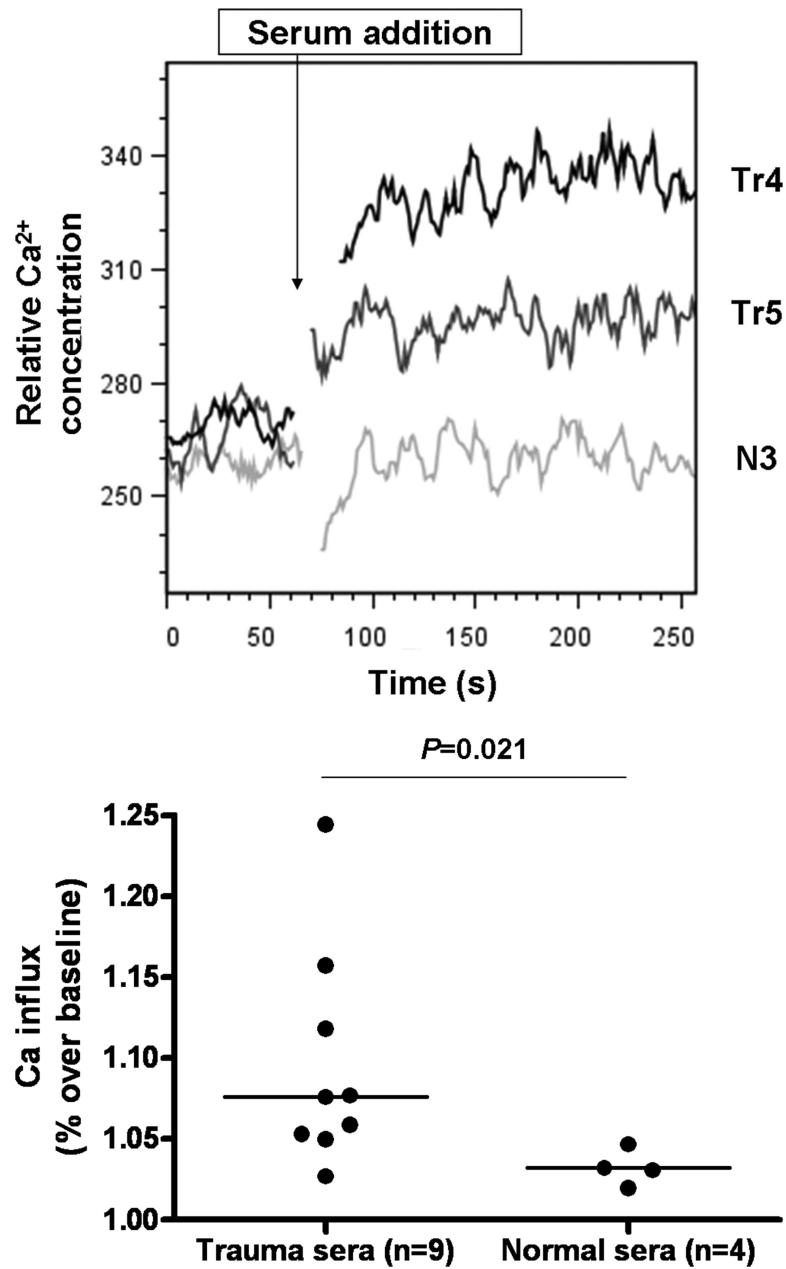
**Figure 5. Ca²⁺ influx into red blood cell after addition of trauma sera**

**A**, Time course of serum-induced changes in intracellular $Ca^{2+}$ of trauma patients (Tr4 and Tr5) or a healthy control (N3) are shown. Arrow indicates the time when trauma or control sera were added to red blood cell preloaded with Fluo-4 AM, 1 minute after start of measurement by flow cytometry. Vertical axis indicates relative intracellular $Ca^{2+}$ concentration, which is estimated from mean fluorescence intensity of Fluo-4 AM. Representative data are shown.

**B**. Cumulative results of $Ca^{2+}$ influx in red blood cells to which trauma sera (n=9) or normal sera (n=4) were added. Vertical axis shows the ratio of mean fluorescence intensity of Fluo-4 AM before and after adding the serum. Horizontal lines indicate the median.
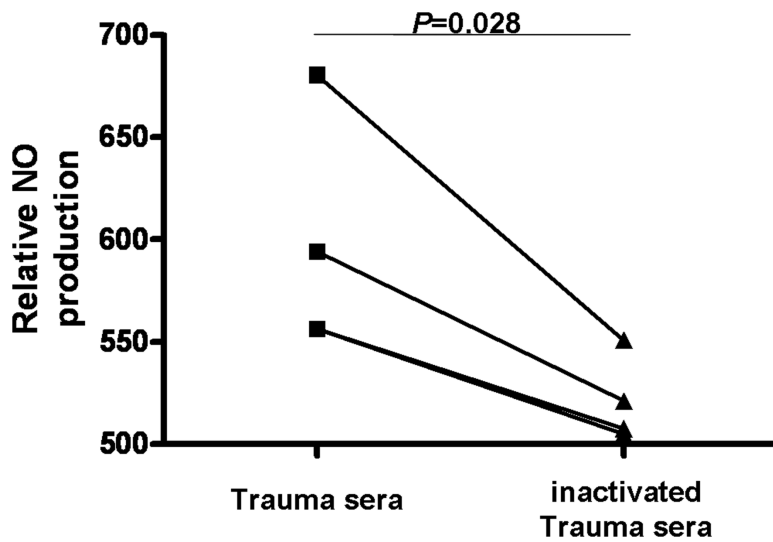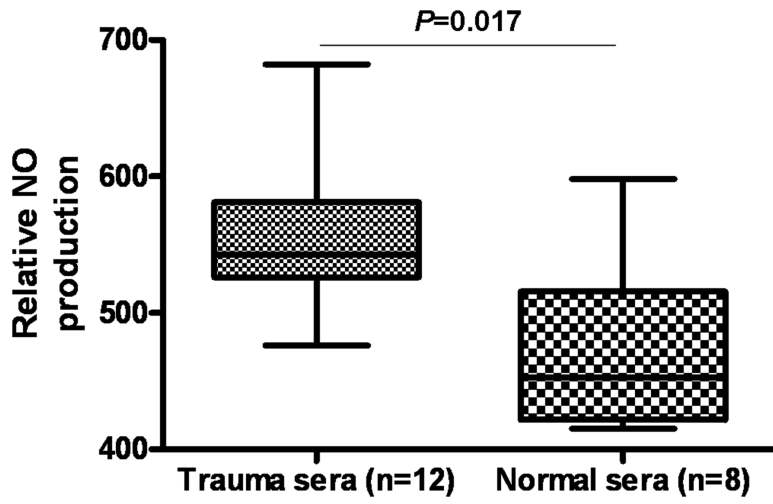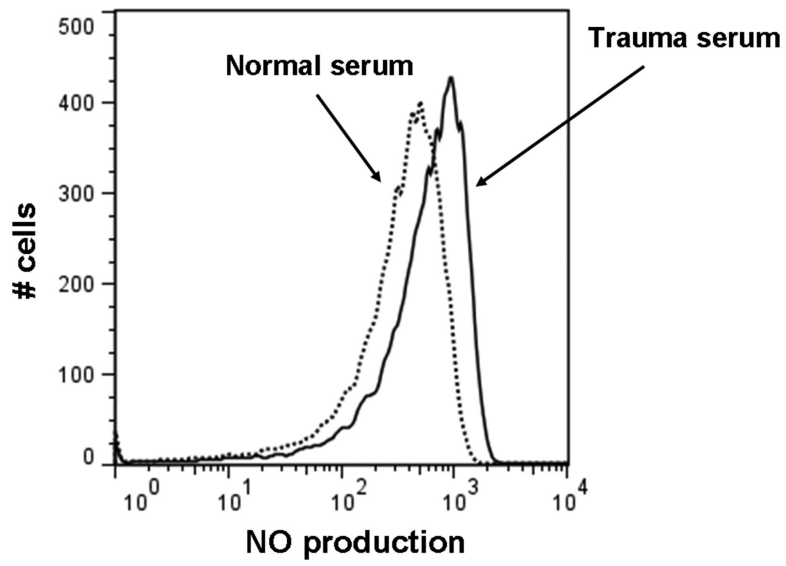
**Figure 6. Nitric oxide production from red blood cells induced by trauma serum**

**A.** Nitric oxide production from healthy red blood cells (type O, Rh negative) measured by flow cytometry after incubation with sera from trauma patients or healthy donors by using DAF-FM diacetate. Representative data is shown.

**B.** Nitric oxide production from red blood cells was expressed as mean fluorescence intensity of DAF-FM diacetate fluorescence, and cumulative data from trauma patients (n=12) or healthy donors (n=8) are shown as box plots. Each box shows the 25th and 75th percentiles. Lines outside the boxes show the lowest and the highest values. Lines inside the boxes show the median.

**C.** 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 minutes. Red blood cells 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 (n=4).

**Table 1**

Patients Demographics

| Demographic data | n=42 |
|---|---|
| Age, mean (±SD), *y* | 46.5 (±19.6) |
| Sex(m/f) | 33/9 |
| Ethnicity, *n*(%) | |
| White | 37(88) |
| Black | 2(5) |
| Hispanic | 1(2) |
| Other | 2(5) |
| Mechanism of Trauma, *n*(%) | *n*=42 |
| MVC | 19(45) |
| Fall | 16(38) |
| PS | 1(2) |
| Other | 6(14) |
| Organ injury, *n*(%) | |
| Bone Fracture | 33(79) |
| Head | 9(21) |
| Lung | 7(17) |
| Spleen | 5(12) |
| Intestine | 3(7) |
| Liver | 0(0) |
| Kidney | 0(0) |
| Other | 1(2) |
| Type of Bone fracture, *n* | |
| Femur | 11 |
| Tibia or Fibula | 10 |
| Spine | 9 |
| Rib | 9 |
| Radius or Ulna | 6 |
| Humerus | 3 |
| Hip | 1 |
| Other | 21 |
| Operative procedure, *n* | |
| Orthopedic surgery | 23 |
| Thoracic surgery | 4 |
| ISS, mean (±SD) | 19.6 (±12.6) |

MVC: motor vehicle crash, PS: pedestrian struck, ISS: Injury Severity Score, SD: standard deviation

**Table 2**

Patient Demographics of C4d deposition on trauma red blood cell

| | Trauma Patients n=40 | Healthy Controls n=17 | P value(Mann-Whitney U test[a], Fisher's Exact Test[b]) |
|---|---|---|---|
| Age, median (interquartile range), *y* | 47 (31-50) | 36(34-38) | 0.32[a] |
| Ethnicity, White, *n* (%) | 36 (90) | 10(59) | 0.011[b] |
| Sex, male, *n* (%) | 31 (78) | 10(59) | 0.13[b] |
| MFI of C4d level , median (interquartile range) | 565(443-886) | 386(309-489) | <0.01[a] |

MFI: mean fluorescence intensity