Leukocyte deformability is a novel biomarker to reflect sepsis-induced disseminated intravascular coagulation

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Abstract

[Aim] Sepsis-induced disseminated intravascular coagulopathy (DIC) is associated with a high mortality rate. The function and deformability of polymorphonuclear leukocytes (PMN) are changed in patients with sepsis. The goal of this study was to characterize the changes in PMN deformability in patients with sepsis-induced DIC and to evaluate the relationship between the severity of DIC and PMN deformability. **[Methods]** 35 patients with sepsis-induced DIC at our department were enrolled in this study. These patients had severe sepsis and an acute DIC score ≥ 4. Blood samples were obtained from these patients on days 1, 3, 7, and PMN deformability and PMN activity, represented as CD11b, was measured with flow cytometry. As contrast, 14 patients who fulfilled with sepsis criteria but not complicated DIC were also entered this study. **[Results]** There was a significant correlation in all patients with sepsis-induced DIC between JAAM DIC score and PMN deformability, also CD11b expression. PMN became more stiffened and CD11b expression was higher in patients with sepsis-induced DIC compared to patients without DIC. **[Conclusion]** PMN deformability correlated with the severity of sepsis-induced DIC and the response to treatment for DIC.

Key words: coagulopathy, sepsis, Leukocyte deformability, disseminated intravascular coagulation, CD11b

INTRODUCTION

Sepsis-induced disseminated intravascular coagulopathy (DIC) is associated with a high mortality rate [1]. Therefore, control of DIC is important to improve outcomes for patients with sepsis. In Japan, the Japanese Association for Acute Medicine (JAAM) has published a scoring system for DIC [2]. The JAAM DIC scoring system is useful for diagnosis and treatment of DIC in the early phase of sepsis [3]. However, the treatment of sepsis-induced DIC controversial and the Surviving Sepsis Campaign Guideline (SSCG) 2012 does not give specific recommendations for the selection criteria and timing of drug administration for patients with this disorder [4].

DIC involves inflammation induced by activated polymorphonuclear leukocytes (PMNs) [5-7]. These activated PMNs can injure vascular endothelial cells and encourage platelet adhesion and agglutination [5], thereby altering coagulation. In addition, coagulation factors, such as tissue factor, factor VIIa, factor Xa and thrombin, activate inflammation through protease-activated receptors (PARs) [6]. Thus, the crosstalk between coagulation and inflammation can increase the severity of sepsis-induced DIC [7].

Infection can alter PMN deformability [8, 9]. PMN stiffening can be induced by granulocyte colony-stimulating factor (G-CSF) [8] and by various pathologic conditions, such as sepsis, trauma, and adult respiratory distress syndrome (ARDS) [10]. In addition, PMN deformability decreases as sepsis progresses [9]. A decrease in PMN deformability can lead to microcirculation failure and organ failure. However, the relationship between PMN deformability and DIC remains poorly understood.

The goal of this study was to investigate changes in PMN deformability in patients with sepsis-induced DIC and to evaluate the relationship between severity of DIC and PMN deformability.

PATIENTS AND METHODS

Patients

This study was approved by the institutional review board of Juntendo University Urayasu Hospital, and informed consent was obtained from each patient or their close relative.

This investigation was a prospective study of patients who were diagnosed with sepsis according to standard criteria [4] and who were treated in the Department of Emergency and Critical Care Medicine, Juntendo University Urayasu Hospital between January 2010 and March 2013. In addition to the criteria for severe sepsis, we choose patients who fulfilled the criteria of the JAAM DIC scoring system. Exclusion criteria were children (<15 years old), history of cancer, and patients for whom sufficient data were not available. Healthy volunteers and patients with sepsis that was not complicated by DIC (sepsis without DIC) were used for comparison purposes.

All patients were treated according to the strategy of the SSCG [4]. There was no predefined protocol regarding the definite indications for sepsis-induced DIC treatment internationally. According to previous small studies [11, 12], recombinant human soluble thrombomodulin (rhTM) and/or antithrombin concentrate were used at the discretion of the attending physician. rhTM was principally administered

intravenously at a dose of 0.06 mg/kg per day, and the infusion was continued for 6 days. Antithrombin concentrate was principally administered intravenously at a dose of 1500 units per day, and the infusion was continued until antithrombin increased by 70%, (maximum, 3 days).

Blood samples

Blood samples from patients were drawn via an arterial line placed after informed consent was obtained. Two milliliters of whole blood with heparin solution (1,000 units/mL: 1.0 part heparin solution to 9.0 parts blood) was taken via a radial arterial line within the first 24 hours after admission and on days 3 and 7 to measure PMN deformability (reflected by CD11b). These biomarkers were measured as soon as possible.

Measurements

PMN deformability and blood rheology

We used a microchannel flow analyzer (MCFAN, MC Healthcare Inc., Tokyo, Japan) to evaluate PMN deformability and blood rheology. The system allows microscopic observation of the flow behavior of blood cells using an in vitro model of a capillary, and is reported elsewhere [8, 9, 13]. A blood sample (200 μL) was vacuumed automatically and was passed through a silicon tip in which 8,736 parallel channels of equal diameter (7 μm) were carved. PMN flow in the channels was monitored and recorded with an inverted microscope and a video camera system. Also the transit time

of the blood sample across the microchip were recorded automatically. To quantify PMN deformability, two parameters were used: transit flow (TF), was defined as the flow per second for 100 μL of whole blood to pass through the microchannel apparatus. When the PMN deformability was so low that the PMN could not pass, we measured the flow through the microchannels in a 3-minute period. Next parameter was defined as the number of obstructed microchannels (NOM) blocked by stiffened PMNs per microscopic field at 1 min. We counted blocked channels in five fields, and the average number of obstructed channels was determined as the NOM.

PMN activation

PMN activation was assessed by measuring the abundance of CD11b on the cell surface, as described in previous studies [14]. To assess CD11b on the surface of PMN, 50 μL of heparinized whole blood was diluted with 175 μL of Hank's Balanced Salt Solutions (HBSS) (Thermo Scientific, Agawarm, MA, USA) and marked with Phycoerythrin-Conjugated anti-human CD11b antibodies (BD Biosciences, San Joes, CA, USA) with incubation and fixation shown as previous report [14], and analyzed by flow cytometry (FACS Calibur, BD Biosciences). Data analysis was performed with FlowJo (Tree Star, Inc., Ashland, OR, USA).

Clinical Parameters

To estimate the severity of severe sepsis and the degree of organ dysfunction, the Acute Physiology and Chronic Health Evaluation II (APACHE II) score and the

Sequential Organ Failure Assessment (SOFA) score were determined. These scoring methods are described in detail elsewhere [15, 16].

We calculated the JAAM DIC scores recorded on the day samples were taken from patients. Scoring systems for the JAAM DIC definitions are found elsewhere [3]. DIC was deemed present when the JAAM DIC score is > 4. Changes in serum concentrations of C-reactive protein (CRP) over time were determined by latex turbidimetric immunoassay with a commercially available CRP kit (Eiken Chemical Co., Tokyo, Japan) to evaluate changes in inflammatory responses. White blood cell (WBC) count was determined with an automated cell counter (CC120, Sysmek, Towa Medical, Hyogo, Japan) for evaluation of WBC kinetics.

Statistics

Descriptive statistics are presented as the mean (interquartile range) or number (percent), as appropriate. Statistical analyses were performed with GraphPad Prism 5 for MAC (GraphPad Software, La Jolla, CA, USA) using the unpaired t test and Spearman's rank correlation coefficient for comparisons of data and Wilcoxon matched-pairs signed rank tests and two-way analysis of variance (ANOVA) test for comparisons of the clinical course. A p value of <0.05 was considered to indicate statistical significance.

RESULTS

Patient characteristics and clinical findings

A total of 3131 patients were admitted to our hospital from the Emergency Department during the study period. Of these, 173 patients fulfilled the criteria for severe sepsis criteria, and 62 of 173 patients had complicated DIC. Of these, 35 patients fulfilled the criteria for inclusion in this study. Fourteen of 111 patients of sepsis without DIC and 21 healthy volunteers were used for comparison purposes.

Characteristics of the patients in this study are shown in Table 1. APACHE II score and SOFA score were significantly higher in sepsis-induced DIC patients than those of sepsis without DIC. There are no significant differences in age, sex, Intensive care units (ICU) stay, mortality between the two groups. The course of the JAAM DIC score, WBC counts and CRP concentrations are shown in Fig. 1. Each data were improving as progression of sepsis treatment in all patients. JAAM DIC score in sepsis-induced DIC patients was significantly higher than those of sepsis without DIC (Fig. 1A, $p<0.0001$).

Quantitative analysis for PMN deformability with MCFAN

Representative video frames illustrating PMN deformability in a typical normal healthy volunteer and a sepsis-induced DIC patient are shown in Fig. 2. In the healthy volunteers (Fig. 2A), the NOM by stiffened PMN ranged from zero to eight after 100 μL of sample blood had passed through the microchip (mean, four per microscopic field), and the TF was 0.44 to 1.73 μL/sec (mean, 1.13 μL/sec). However, for patients with sepsis, PMN were activated and stiffened, so some microchannels were occluded (Fig. 2B). Application of some samples resulted in complete occlusion of all

of the microchannels with activated PMN. As described above, we analyzed PMN deformability quantitatively with these numbers of occluded microchannels and transit flow in a 3-minute period.

Rheology in sepsis-induced DIC patients

Serial change in PMN deformability is shown in Fig. 3. With institution of treatment for sepsis-induced DIC, PMN deformability (assessed by NOM and TF) improved on a daily basis. The relationship between PMN deformability and JAAM DIC score are shown in Fig. 4 and 5. Significant correlation between the NOM (Fig. 4A), TF (Fig. 4B) and JAAM DIC score was seen in all patients with sepsis-induced DIC (NOM; $p<0.005$, TF; $p<0.05$). PMN deformability in patients with sepsis-induced DIC was markedly lower than that in samples from healthy volunteers (Fig. 5, $p<0.0001$). Moreover, NOM was lower in patients of sepsis without DIC than in healthy volunteers (Fig. 5A, $p<0.0001$).

PMN activity in patients with sepsis-induced DIC

To determine the relationship between PMN activity and PMN deformability, the abundance of CD11b on the cell surface was measured in 11 sepsis-induced DIC patients and five patients of sepsis without DIC who were admitted to our hospital between July 2012 and March 2013. Nine healthy volunteers were used as controls. Data are shown in Fig. 6 and 7. A significant correlation between JAAM DIC score and CD11b expression was seen in all patients with sepsis-induced DIC (Fig. 6, $p<0.05$).

CD11b expression in patients with sepsis-induced DIC was more than that in healthy volunteers and those of sepsis without DIC (Fig. 7; vs. healthy volunteers, $p<0.005$; vs. sepsis without DIC, $p<0.01$).

DISCUSSION

Sepsis is one of the most serious problems in the critical care field and is responsible for a quarter of all causes of death worldwide [4]. Shock and DIC are lethal complication of sepsis. The pathophysiologic mechanism of DIC might involve crosstalk between inflammation triggered by activated PMN and coagulopathy triggered by tissue factor at the endothelium. Therefore, the present study investigated the relationship between PMN deformability and coagulopathy in patients with sepsis-induced DIC.

We showed that PMN deformability was significantly lower in patients with sepsis-induced DIC (Fig. 5). Moreover, NOM, TF and PMN activity changed as the JAAM DIC score changed (Fig. 4, 7). These observations suggest that PMN deformability correlated with the severity of sepsis-induced DIC and might correlate with the response to treatment for DIC. Even in the context of a JAAM DIC score < 4, leukocyte rheology might help to diagnose or predict early progression towards DIC in patients with systemic inflammatory response syndrome (SIRS)-associated coagulopathy. Therefore, rheology data in patients with sepsis-induced DIC might be used to guide early institution of various anti-DIC strategies.

Varied methods to measure PMN deformability are reported elsewhere [17,

18], but we used the method described by Tanaka et al. [8, 9, 13], which is able to measure PMN deformability at the bedside easily, quickly, in a serial fashion and in real-time. Therefore, this strategy might lead to earlier diagnosis of DIC, and earlier institution of treatment, and effective monitoring of the response to treatment.

Endotoxin, tumor necrosis factor-α and interleukin (IL) -1β can induce a decrease in PMN deformability [19, 20]. These mediators cause soluble G actin to polymerize to filamentous F actin, resulting in an increase in PMN rigidity and a decrease in PMN deformability [21]. The levels of endotoxin, tumor necrosis factor-α and IL-1β can increase as a result of the crosstalk between coagulation and inflammation in patients with sepsis-induced DIC, thereby inducing a decrease in PMN deformability.

Previously, we reported that PMN deformability was decreased in patients with sepsis or trauma [9]. We found in this study that PMN became more stiffened in patients with sepsis-induced DIC. APACHEII score as well as SOFA score was significantly higher in those patients suggest that microcirculation failure was induced in patients with sepsis-induced DIC.

This study has some limitations. First, this study population was very small. Larger studies are needed to clarify the significance of PMN function in patients with sepsis-induced DIC. Second, this study did not investigate the function of endothelial cells. Endothelial cells are very important for inflammation and coagulation. This study focused only on the function of PMNs. Further study is needed to evaluate the relationship between endothelial function and leukocyte deformability. Lastly, rheology

can vary according to the WBC count, red blood cell count, and platelet count and according to the presence/absence of hyperlipidemia, smoking, and obesity. However, these factors are not thought to have been effected rheology in patients with sepsis-induced DIC in this study. Because patients with sepsis had a large invasion, PMN deformability might have had a big influence on rheology.

In conclusion, PMN deformability reflected the severity of sepsis-induced DIC as well as the response to anti-DIC treatment. For this purpose, MCFAN is a very easy instrument for the rapid and serial bedside evaluation of PMN deformability.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

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Figure legends

Table 1. Patient characteristics

Data are expressed as mean (interquartile range) or number (percent). APACHE II score, acute physiology and chronic health evaluation II score; SOFA score, sequential organ failure assessment score; ICU, intensive care units; UTI, urinary tract infection; GPC, gram positive cocci; GNR, gram negative rods.

Fig. 1. Clinical course of JAAM DIC score, WBC count and CRP concentration

The course of the JAAM DIC score (A), WBC count (B) and CRP concentration (C) are shown. Data are shown as mean + or − standard deviation. JAAM, Japanese Association for Acute Medicine; DIC, disseminated intravascular coagulation; WBC, white blood cell; CRP, C-reactive protein. Closed circle, patients with sepsis-induced DIC; closed square, patients without sepsis-induced DIC; †$p<0.05$, each data vs. day 1; $^{*}p<0.0001$, clinical course of JAAM DIC score in patients with sepsis-induced DIC vs. without sepsis-induced DIC.

Fig. 2. Representative video frames illustrating polymorphonuclear leukocytes deformability

Healthy volunteer (A); sepsis-induced disseminated intravascular coagulation (DIC) patients (B). The black arrow indicates the direction of blood flow.

Fig. 3. Clinical course of polymorphonuclear leukocytes deformability

Data are shown as mean ± standard deviation. TF improved on a daily basis. NOM, number of obstructed microchannels; TF, transit flow.

Fig. 4. Relationship between polymorphonuclear leukocytes deformability and JAAM DIC score

Correlation between JAAM DIC score, NOM (A), and TF (B) are shown. NOM vs. JAAM DIC score; $p<0.005$, $r^2=0.11$, TF vs. JAAM DIC score; $p<0.05$, $r^2=0.033$. The dot line indicates the mean for healthy volunteers. NOM, number of obstructed microchannels; TF, transit flow; JAAM, Japanese Association for Acute Medicine; DIC, disseminated intravascular coagulation.

Fig. 5. Comparison of polymorphonuclear leukocytes deformability in patients with or without sepsis-induced DIC

Data are shown as mean ± standard deviation of NOM (A) and TF (B) in each group. NOM, number of obstructed microchannels; TF, transit flow; DIC, disseminated intravascular coagulation; Closed circle, healthy volunteers; closed square, patients without sepsis-induced DIC; closed triangle, patients with sepsis-induced DIC; $^{*}p<0.0001$, †$p<0.05$, **$p<0.0005$.

Fig.6. Relationship between polymorphonuclear leukocytes activity and JAAM DIC score

Correlation between CD11b expression and JAAM DIC score is shown. JAAM DIC score vs. CD11b; $p<0.05$, $r^2=0.3404$. The dot line indicates the mean for healthy volunteers. JAAM, Japanese Association for Acute Medicine; DIC, disseminated intravascular coagulation.

Fig. 7. Comparison of polymorphonuclear leukocytes activity in patients with or without sepsis-induced DIC

The mean + standard deviation of CD11b expression in each group are shown. DIC, disseminated intravascular coagulation; dot bar; healthy volunteers: checked bar; patients without sepsis-induced DIC: striped bar; patients with sepsis-induced DIC. $\Delta p<0.005$, $***p<0.01$.

Figure 1.

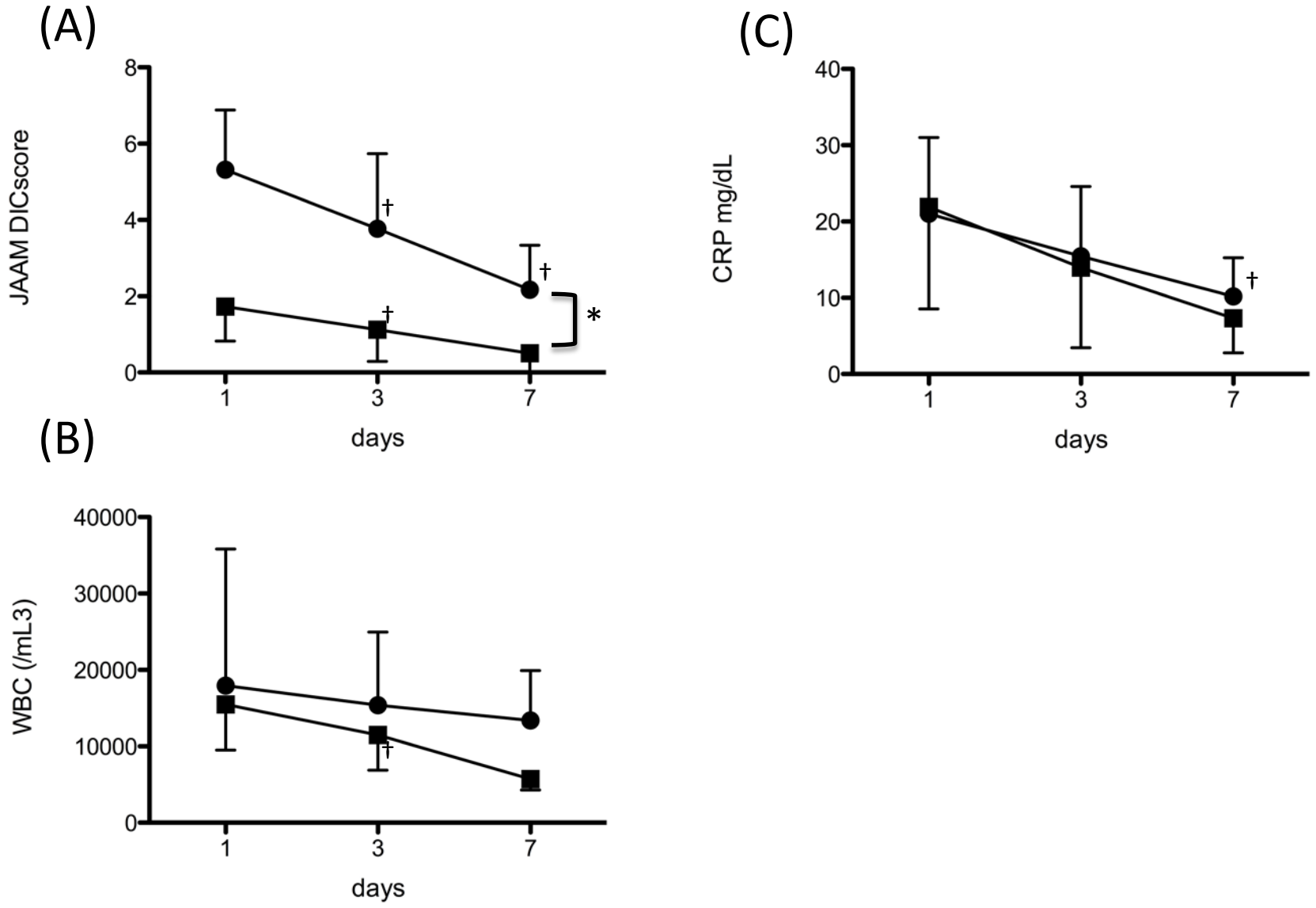
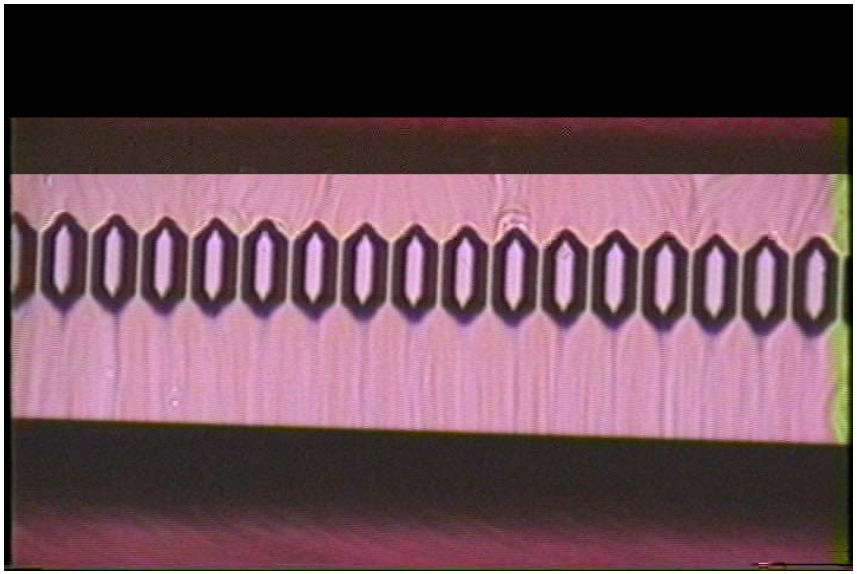

(A)
JAAM DICscore
8
6
4
2
0
1
3
7
days
†
†
†
*
(C)
CRP mg/dL
40
30
20
10
0
1
3
7
days
†
(B)
WBC (/mL3)
40000
30000
20000
10000
0
1
3
7
days
†

Figure 2.

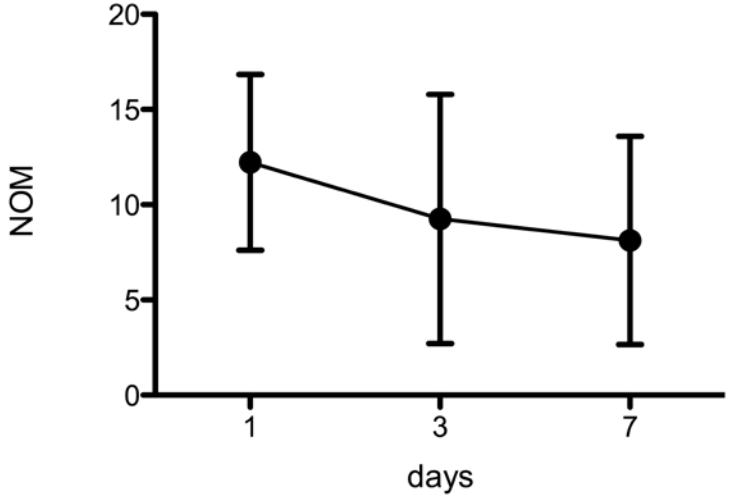
A: Healthy volunteer



B: Sepsis-induced DIC

Figure 3.

(A)



(B)

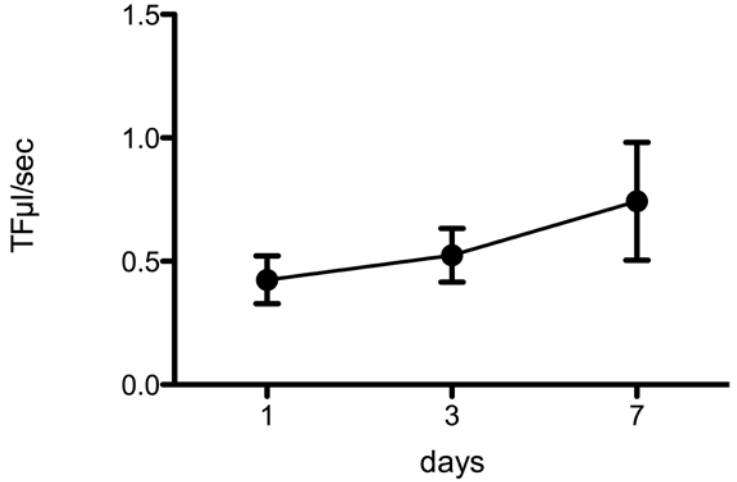
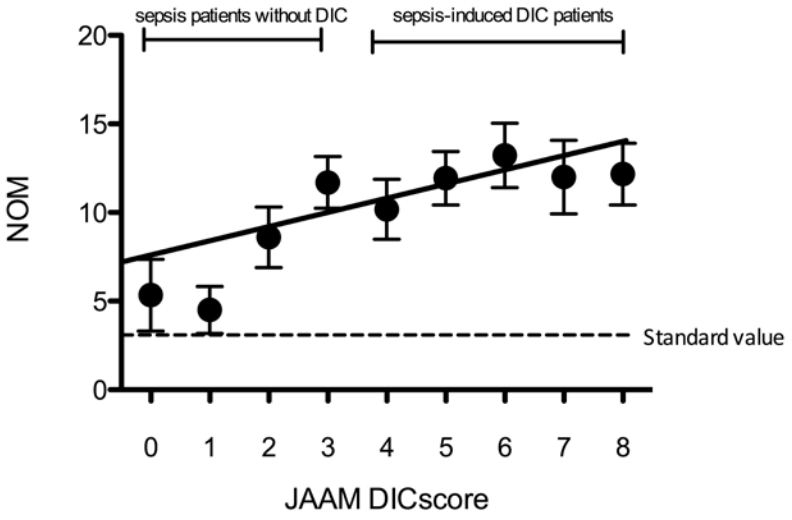


Figure 4.

(A)


sepsis patients without DIC
sepsis-induced DIC patients
NOM
20
15
10
5
0
Standard value
0 1 2 3 4 5 6 7 8
JAAM DICscore

(B)

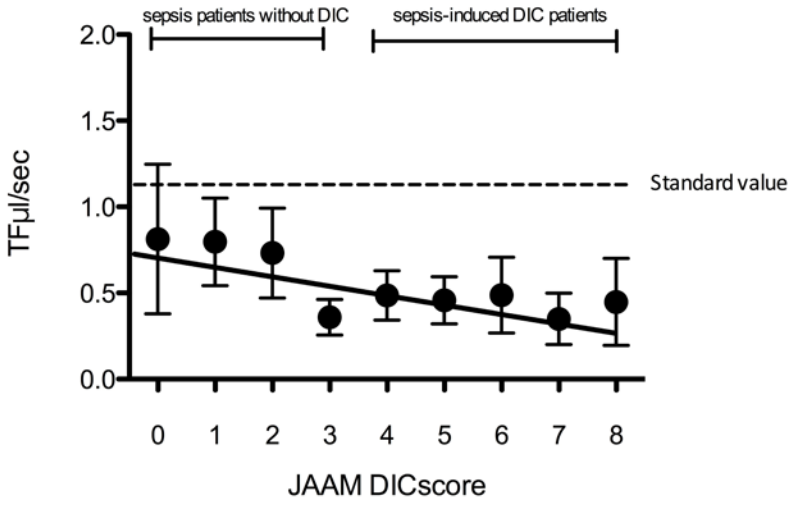

sepsis patients without DIC
sepsis-induced DIC patients
TFμl/sec
2.0
1.5
1.0
0.5
0.0
Standard value
0 1 2 3 4 5 6 7 8
JAAM DICscore

Figure 5.

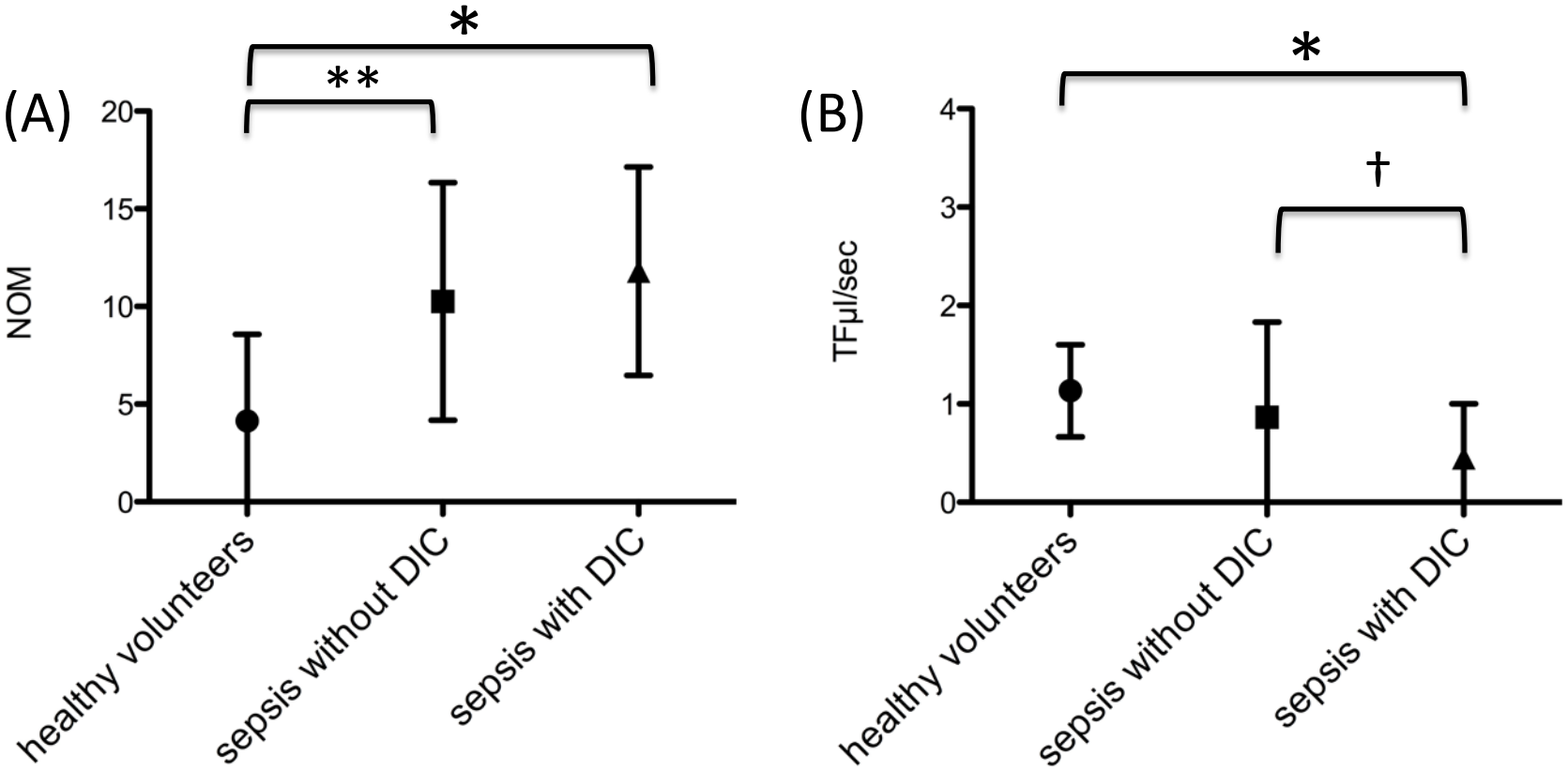

(A)
NOM
20
15
10
5
0
*
**
healthy volunteers
sepsis without DIC
sepsis with DIC
(B)
TFμl/sec
4
3
2
1
0
*
†
healthy volunteers
sepsis without DIC
sepsis with DIC

Figure 6.

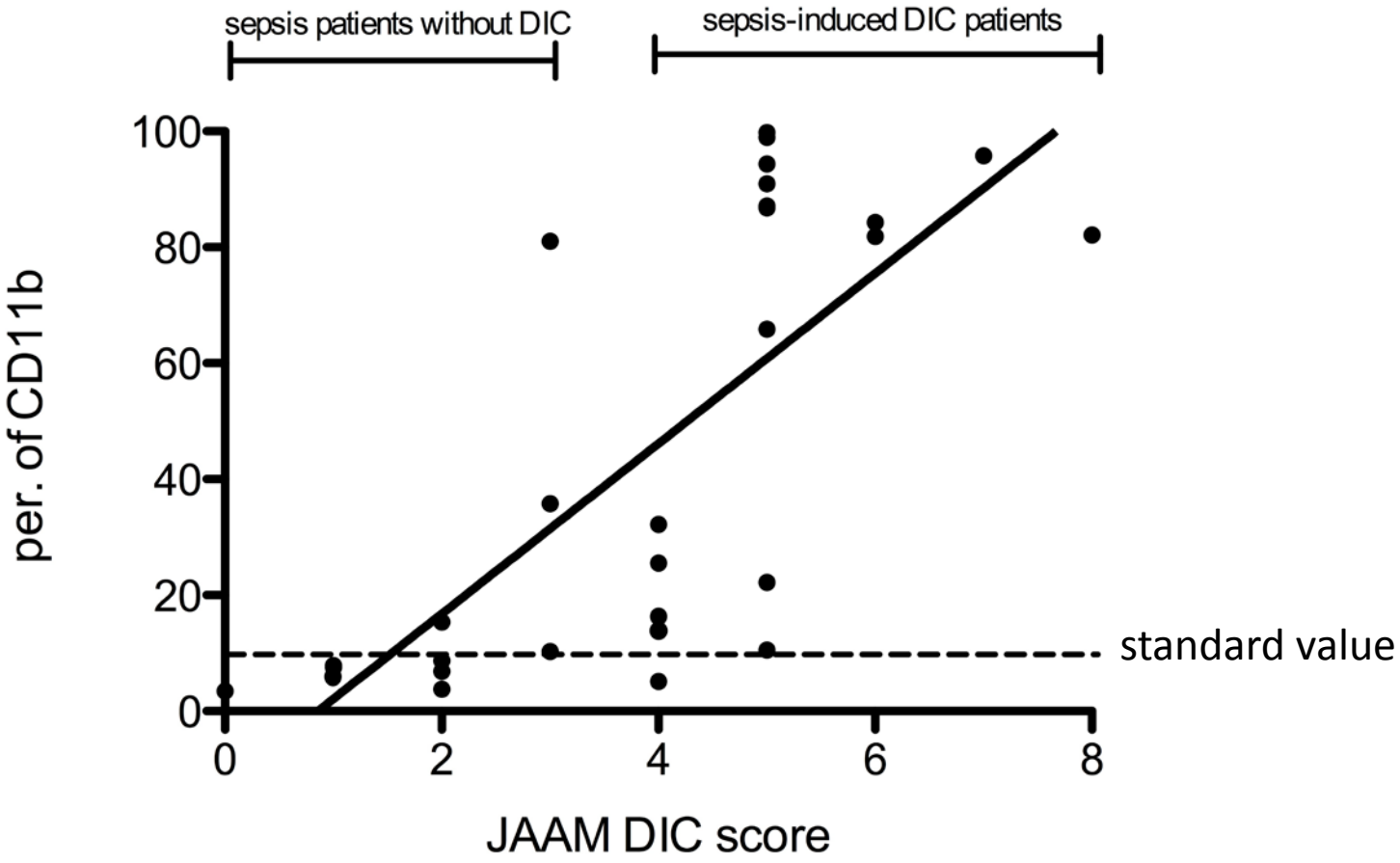

sepsis patients without DIC
sepsis-induced DIC patients
per. of CD11b
100
80
60
40
20
0
0
2
4
6
8
JAAM DIC score
standard value

Figure 7.

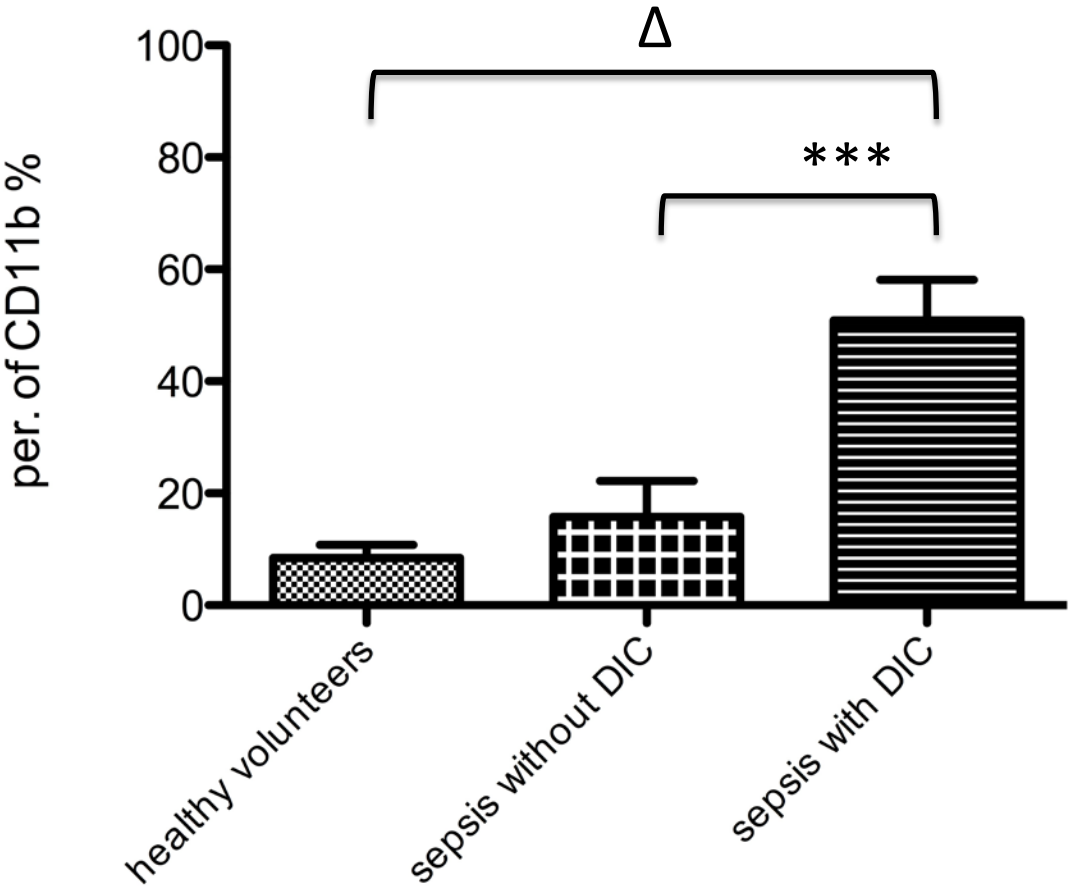

Δ
***
per. of CD11b %
100
80
60
40
20
0
healthy volunteers
sepsis without DIC
sepsis with DIC

table 1.

	sepsis with DIC (n=35)	sepsis without DIC (n=14)	*p* value
age	67±14 (31-92)	65±16 (27-89)	0.69
sex (M/F)	25/10	7/7	0.19
APACHE Ⅱ score	26±8 (9-46)	20±6 (8-32)	0.017
SOFA score	10±3 (3-18)	4±3 (1-11)	p<0.0001
ICU stay	10±7 (1-31)	10±6 (2-22)	0.79
mortality	11 (31%)	2 (14%)	0.29
Source of sepsis			0.0012
UTI	11	2	
pneumonia	8	3	
abdominal	8	0	
soft tissue	2	8	
others	6	1	
Causative bacterial agent			0.82
GPC	14	5	
GNR	16	6	
others	5	3	

Table 1. Patient characteristics

Data are expressed as mean (interquartile range) or number (percent). APACHE II score: acute physiology and chronic health evaluation II score, SOFA score: sequential organ failure assessment score, ICU: intensive care units, UTI: urinary tract infection, GPC: gram positive cocci, GNR: gram negative rods.