Sera from Septic Patients Contain the Inhibiting Activity of the Extracellular ATP-Dependent Inflammasome Pathway

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Running title: Septic sera suppress the immunogenicity of extracellular ATP

Abstract

Immunoparalysis is a common cause of death for critical care patients with sepsis, during which comprehensive suppression of innate and adaptive immunity plays a significant pathophysiological role. Although the underlying mechanisms are unknown, damage-associated molecular pattern molecules (DAMPs) from septic tissues might be involved. Therefore, we surveyed sera from septic patients for factors that suppress the innate immune response to DAMPs, including adenosine triphosphate (ATP), monosodium urate, and high mobility group box-1. Macrophages, derived from THP-1 human acute monocytic leukemia cells, were incubated with each DAMP, in the presence or absence of sera that were collected from critically ill patients. Secreted cytokines were then quantified, and cell lysates were assayed for relevant intracellular signaling mediators. Sera from septic patients who ultimately did not survive significantly suppressed IL-1β production only in response to extracellular ATP. This effect was most pronounced with sera collected on day 3, and persisted with sera collected on day 7. However, this effect was not observed when THP-1 cells were treated with sera from survivors of sepsis. Septic sera collected at the time of admission (day 1) also diminished intracellular levels of inositol 1,4,5-triphosphate and cytosolic calcium ($P < 0.01$), both of which are essential for ATP signaling. Finally, activated caspase-1 was significantly diminished in cells exposed to sera collected on day 7 ($P < 0.05$). In conclusion, the sera of septic patients contain certain factors that persistently suppress the immune response to extracellular ATP, thereby leading to adverse clinical outcomes.

Keywords: damage-associated molecular patterns; extracellular adenosine triphophate; immunoparalysis; innate immune response; sepsis

Introduction

Sepsis, a systemic inflammatory response to infection, causes multi-organ failure, severe morbidity, and death (Liu et al. 2014; Kissoon and Uyeki 2016); endothelial dysfunction, metabolic changes, and overwhelming or hyperinflammatory innate immune responses to pathogen-associated molecular patterns have been proposed to be the main underlying mechanisms of this condition. Sepsis-induced immunoparalysis, in which both innate and adaptive immunity are suppressed (Frazier and Hall 2008; Hotchkiss et al. 2013), is also an active focus of research, since it increases the risk of secondary infections and promotes adverse clinical outcomes (Hotchkiss et al. 2013). Of note, Fattahi and Ward (2017) identified universal mechanisms that drive sepsis-induced immunosuppression, including reduced antigen processing by innate immune cells, the accumulation of regulatory T cells, and the abundant production of immunosuppressive factors.

Inflammatory responses mediated by innate immunity generally promote healing and microbial clearance, although the massive production of pro- and anti-inflammatory cytokines by macrophages might also lead to pathological inflammation. Primary inflammatory cytokines include tumor necrosis factor-α, interleukin-1β (IL-1β), and IL-6, which mediate acute inflammation (Trikha et al. 2003) but also cause cell injury and cell death (Kim et al. 2011). In turn, injured tissues and dying cells generate damage-associated molecular patterns (DAMPs), which then act on various inflammasomes to promote or suppress inflammation (Guo et al. 2015). However, the roles of these DAMPs in sepsis are unknown.

DAMPs stimulate nucleotide oligomerization domain-like receptor (NLR) inflammasomes to activate caspase-1, which then cleaves pro-IL-1β to generate mature, bioactive IL-1β (Chen and Nuñez 2010; Takeuchi and Akira 2010). One such inflammasome is NLR protein 3 (NLRP3), which contains an ASC-PYCARD domain (Cai et al. 2014; Lu et al. 2014) and is particularly sensitive to a wide variety of DAMPs including adenosine triphosphate (ATP), crystalline substances, nucleic acids, and hyaluronan (Lamkanfi and Dixit 2014; Vanaja et al. 2015). In this study, we screened sera from septic patients for the ability to alter innate immune responses to DAMPs and inflammasomes, with the goal of defining mechanisms that drive immunoparalysis during sepsis.

Materials and Methods

Study design and collection of human sera

This study was approved by the Ethics Committee Review Board of Hiroshima University, and was compliant with the Declaration of Helsinki regarding the review and publication of patient data. Sera were collected from patients admitted to the Department of Emergency and Critical Care Medicine, Hiroshima University Hospital for sepsis, with consent from patients or from a family member. Sepsis was diagnosed according to the American College of Chest Physicians and the Society of Critical Care Medicine Consensus Conference (Singer et al. 2016). For comparison, sera were also collected with consent from patients > 50 years of age who suffered trauma from any cause, as well as from five healthy volunteers. On the day of admission (day 1), as well as day 3 and

day 7, peripheral blood samples were collected into evacuated non-EDTA VENOJECT® II tubes (Terumo, Somerset, NJ, USA) through an indwelling arterial line or a peripheral vein. Blood samples were allowed to clot for ≥ 30 min at room temperature, and sera were obtained by centrifugation at 3,000 $\times g$ for 10 min and then stored at −80 °C for further analysis.

Reagents

Human acute monocytic leukemia THP-1 monocytes (TIB-20) were obtained from the American Type Culture Collection (Manassas, VA, USA). Phorbol 12-myristate 13-acetate, ATP, and monosodium urate crystals were purchased from Sigma-Aldrich (St. Louis, MO, USA). High mobility group box 1 (HMGB1) was obtained from Thermo Fisher Scientific (Illkirch, France), and trypsin-EDTA was acquired from Gibco/Thermo Fisher (Burlington, ON, Canada).

Human inflammatory cytokine panel

Sera were assayed for 13 inflammatory cytokines using a Legendplex™ human inflammation panel (BioLegend, San Diego, CA, USA), following the manufacturer's protocol. The panel consists of bead-based immunoassays, similar to sandwich immunoassays, with the lowest limit of quantification being approximately 0.6 pg/mL. Data were acquired using a BD FACSCalibur™ (Becton, Dickinson Biosciences, San Jose, CA, USA) and analyzed with Legendplex™ Data Analysis Software (BioLegend).

Cell culture and response to DAMPs

THP-1 monocytes were seeded at 1×10^5 cells/well in 24-well plates containing RPMI 1640 (Gibco) media with 10 % fetal bovine serum (Gibco) and 100 U/mL penicillin/streptomycin (Thermo Fisher) and differentiated for 48 h with 0.5 μM phorbol 12-myristate 13-acetate. Media were then removed, and cells were exposed to sera diluted 60×, with or without DAMPs including 2.0 mM ATP (Laliberte et al. 1999), 200 μg/mL monosodium urate (Gicquel et al. 2015), and 1 μg/mL HMGB1 (Robert et al. 2010); this incubation proceeded for 8 h. The resulting culture supernatant was collected, centrifuged at 40,000 rpm for 10 min at 4 °C, and transferred to 1.5-mL centrifuge tubes for immediate quantification of IL-1β or stored at −20 °C for further experiments. For the vehicle control condition, THP-1 macrophages were incubated with serum-free RPMI without supplementation (untreated). In addition, cells were washed twice with cold phosphate-buffered saline (Gibco) and collected by scraping them into cell lysate buffer (Abcam, Cambridge, U.K.) following the product instructions. Lysates were then cleared of debris and transferred to 1.5-mL centrifuge tubes for immediate quantification of intracellular signaling mediators or stored at −20 °C for further experiments.

Cell viability

Cell viability was assessed by annexin V/propidium iodide staining (eBioscience, San Diego, CA, USA). Briefly, cells were harvested with trypsin-EDTA, suspended in staining buffer, stained

for 20 min with annexin V and propidium iodide, and analyzed using a BD FACSCalibur™ and BD CellQuest™ Pro (Becton, Dickinson Biosciences, San Jose, CA, USA).

$P2X_7$ channel uptake

YO-PRO-1, a 629-Da fluorescent nucleic acid dye that is transported across the cell membrane by $P2X_7$, was used to quantify the uptake of extracellular ATP, as previously described (Cankurtaran-Sayar et al. 2009). In brief, cells were collected with trypsin-EDTA, centrifuged at 1,500 rpm for 5 min, washed once with cold phosphate-buffered saline, and stained with 2 μM YO-PRO-1 (Invitrogen, Carlsbad, CA, USA) for 30 min in the dark. The mean fluorescence intensity was then analyzed using a BD FACSCalibur™ and BD CellQuest™ Pro (Becton, Dickinson Biosciences, San Jose, CA, USA).

Quantification of cytokines and mediators of intracellular signaling

IL-1β, intracellular pro-IL-1β, and caspase-1 p20 were quantified using a BD OptEIA™ Set for human IL-1β (Becton, Dickinson Biosciences), a Human Pro-IL-1β/IL-1F2 Immunoassay Kit (R&D Systems, Minneapolis, MN, USA), and a Human Caspase-1/ICE Immunoassay Kit (R&D Systems, Minneapolis, MN, USA), respectively. All three kits are based on sandwich enzyme-linked immunosorbent assays and were used according to the manufacturer's instructions. Inositol 1,4,5-triphosphate (Cusabio, Wuhan Hi-tech Medical Devices Park, Wuhan, China) and cyclic AMP (R&D Systems, Minneapolis, MN, USA) were quantified by competitive

enzyme-linked immunosorbent assays. For all assays, readings at 450 nm were obtained using a spectrophotometer (Perkin Elmer, Courtaboeuf, France). Cytosolic calcium was quantified using the Calcium Detection Assay Kit (Abcam), in which a chromogenic complex that is formed between calcium ions and O-cresolphthalein was quantified by spectrophotometry at 575 nm using a Varioskan Flash (Thermo Scientific, Illkirch, France).

ATP assays

Extracellular and intracellular ATP levels were quantified using an ATP Detection Kit (Invitrogen) according to the manufacturer's instructions. Readings at 450 nm were obtained using a spectrophotometer (Perkin Elmer, Courtaboeuf, France).

Statistical analysis

Data were analyzed using SPSS 22 (IBM, Armonk, NY, USA). Categorical variables were compared by the Fisher's exact test, whereas multiple groups of normally distributed, unpaired data on an interval scale were tested by a one-way analysis of variance, and then compared pairwise by *post hoc* analysis. A Mann–Whitney's *U* test or χ^2 test was used for pairwise comparison of non-normally distributed, unpaired data on an interval scale. $P < 0.05$ was considered statistically significant. Continuous variables are reported or plotted in bar graphs as the mean ± standard deviations, with bold lines indicating median values, boxes indicating interquartile ranges, and whiskers indicating extreme values within 2.5-fold of the interquartile range around the median.

Results

Cytokine and chemokine profiles of sera from septic patients

The characteristics of critically ill patients and healthy volunteers are listed in Table 1. Septic patients were further stratified as shown in Table 2. Because of inflammation, serum levels of C-reactive protein and lactate at the time of patient admission (day 1) were significantly higher in septic patients than in trauma patients, as previously observed during the acute phase (Póvoa 2002; Jain et al. 2011; Jin et al. 2014; Markanday 2015). Similarly, IL-1β, IL-6, IL-8, IL-10, IL-18, interferon-α, macrophage inflammatory protein-1, and tumor necrosis factor-α were significantly elevated in sera from septic patients as compared with those in sera from trauma patients (Fig. 1); in contrast, interferon-γ, IL-23, IL-12, IL-17, and IL-33 were barely detectable in < 30% of all serum samples (data not shown). These observations are concordant with previous studies on innate immunity during acute-phase sepsis, during which pro- and anti-inflammatory responses were both found to be exaggerated (Póvoa 2002; Giannoudis et al. 2004; Kirchhoff et al. 2009; Jaffer et al. 2010; Saraiva and O'Garra 2010; Jain et al. 2011; Jin et al. 2014; Reikeras et al. 2014).

DAMP-specific anti-inflammatory activity of sera from septic patients

After exposing THP-1 macrophages to patient sera, the resulting culture supernatant was tested for IL-1β levels. Strikingly, sera collected from septic patients at the time of admission tended to suppress the *in vitro* production of IL-1β by macrophages; however, sera from trauma

patients or healthy volunteers did not (Fig. 2A). This result suggests that septic sera, collected at the time of patient admission, are likely predominantly anti-inflammatory, despite enhanced levels of both pro- and anti-inflammatory cytokines. Further, upon stimulation with each known DAMP in the presence of patient sera, IL-1β production was suppressed only in response to extracellular ATP (Fig. 2B), but not in response to monosodium urate or HMGB1 (Fig. 2C, D). Similar analysis of septic sera collected on days 3 and 7 after admission indicated prominent suppression of ATP-induced IL-1β production by sera collected on day 3. This activity persisted with sera collected on day 7 from patients who ultimately did not survive; however, this was not the case when using sera collected on day 7 from patients who survived (Fig. 3A). As with sera collected on day 1, day 3, and day 7, these trends were not observed in macrophages stimulated with monosodium urate (Fig. 3B). The observed loss of ATP-induced IL-1β production was not due to serum-induced apoptosis, as cell viability after 8 h was comparable among all cells (data not shown). Therefore, we hypothesize that sera from septic patients have inherent anti-inflammatory functions and might contain factor(s) that suppress the response to extracellular ATP.

Possible anti-inflammatory mechanisms of septic sera

Extracellular ATP is known to trigger IL-1β production in macrophages by acting on NLRP3 inflammasomes via purinergic pathways that depend on P2X, P2Y, and adenosine P1 receptors (Baron et al. 2015). For example, extracellular ATP binds $P2X_7$ and thereby elicits cytosolic K^+ efflux and extracellular Na^+/Ca^{2+} influx. Of note, $P2X_7$ also transports large molecular weight tracers

into cells, including YO-PRO-1, at millimolar extracellular ATP concentrations. ATP and adenosine diphosphate also act through P2Y, thereby generating inositol 1,4,5-triphosphate and boosting cytosolic calcium. Finally, adenosine, a product of ATP hydrolysis, acts through adenosine receptors to form cyclic AMP, which in turn suppresses NLRP3. In any case, NLRP3 activation by purinergic receptors activates caspase-1, which processes pro-IL-1β into mature IL-1β. Hence, we hypothesized that the observed loss of ATP-induced IL-1β production could be due to ATP hydrolysis by ATPases in sera from septic patients, inhibition of $P2X_7$, or inhibition of NLRP3.

ATPase activity in sera from septic patients is negligible

Using a luciferase-based assay, we compared ATP levels in supernatants after incubating macrophages with ATP and the sera from healthy donors, trauma patients, or septic patients. We found that ATP levels were similar among these groups (Fig. 4A). In addition, residual ATP in sera probably did not affect macrophage activity since these samples were diluted such that ATP levels would be negligible before addition to cells (Fig. 4B). These results suggest that ATPase activity in patient sera, if any, did not significantly affect IL-1β production in response to extracellular ATP.

Septic sera from non-survivors activates the $P2X_7$ channel

As previously reported, the uptake of YO-PRO-1 or other large, positively charged tracers is a direct measure of $P2X_7$ activity (Cankurtaran-Sayar et al. 2009; Baron et al. 2015). Thus, THP-1 macrophages stimulated with ATP and patient sera were stained with YO-PRO-1 and analyzed by

flow cytometry. The mean fluorescence intensity was comparable among macrophages treated with sera from healthy controls, trauma patients, or septic patients (Fig. 4C). However, this was higher in macrophages treated with sera collected on days 1 and 7 from patients who did not survive than in cells treated with sera from septic patients who survived ($P < 0.05$; Fig. 4D). In the presence of 10 μM A-740003, a specific inhibitor of $P2X_7$, the mean fluorescence intensity was dramatically diminished in all cells including those treated with sera from non-survivors (data not shown). These observations suggest that septic sera from non-survivors enhance $P2X_7$ activity.

Inhibition of pro-inflammatory pathways by septic sera

Cell lysates were then subjected to the detection of cytosolic mediators associated with the ATP-dependent inflammasome pathway after exposure to ATP in the presence of patient sera. Intracellular ATP was slightly lower in macrophages treated with sera collected from septic patients on day 1 than in cells treated with sera from trauma patients (Fig. 5A). Accordingly, levels of inositol 1,4,5-triphosphate and cytosolic calcium were significantly lower in the former group than in the latter, as well as compared to those in cells exposed to sera from healthy controls ($P < 0.01$; Fig. 5B, C). However, these effects were largely absent in cells treated with sera collected on day 7 from septic patients. In contrast, cyclic AMP levels were comparable among all cell groups (Fig. 5D). Collectively, these data indicate that sera collected from septic patients at the time of admission suppress pro-inflammatory pathways upstream of NLRP3.

Sera from septic patients who did not survive suppress caspase-1 activation

Pro-IL-1β, which is processed to form IL-1β following NLRP3 and caspase-1 activation, was slightly more abundant in cells treated with sera from septic patients collected on days 1 and 7 than in cells exposed to sera from healthy controls or trauma patients (Fig. 6A). Conversely, active caspase-1 was significantly less abundant in macrophages treated with sera from septic patients collected on day 1 ($P < 0.01$), but not in cells stimulated with sera collected on day 7 from septic patients (Fig. 6B). However, the significant decrease in active caspase-1 was also observed in cells treated with sera collected on day 7 from patients who did not survive ($P < 0.05$; Fig. 6C). Similar trends were observed in terms of intracellular IL-1β levels (Fig. 6D, E). Taken together, these data suggest that activation of NLRP3 and caspase-1 in response to extracellular ATP is suppressed by factors that are present in sera from septic patients. This suppressive effect is clearly associated with prolonged immunoparalysis and poor clinical outcomes since it was found to be more pronounced in sera collected from patients who did not survive.

Discussion

Our data indicated that sera from septic patients suppress IL-1β production in macrophages stimulated with extracellular ATP, likely by antagonizing upstream NLRP3 or pro-inflammatory pathways. This suppressive effect peaked on day 3 after admission and persisted until day 7 among patients who did not survive, but was lost by day 7 among patients who survived. These observations suggest a new mechanism through which sepsis mediates

immunoparalysis, and partially support the prior hypothesis that persistent suppression of innate and adaptive immunity can cause late-stage death in septic patients (Hotchkiss et al. 2013).

Inflammation and cell death in critically ill patients results in the abundant generation of DAMPs, which induce pro-inflammatory cytokine production (Cauwels et al. 2014; Stoffels et al. 2015; Timmermans et al. 2016). Of the known DAMPs, extracellular ATP is one of the most potent. However, extracellular ATP might also suppress inflammation since its hydrolytic products are uniquely immunosuppressive (Borsellino et al. 2007; Beavis et al. 2012). In particular, ATP acts through purinergic P2 receptors (either the P2X cation-selective receptor channels or the P2Y metabotropic G protein-coupled receptors), thereby rapidly depleting regulatory T cells (Aswad et al. 2005), or modulating T cell activity (Ledderose et al. 2016). Conversely, hydrolytic products of ATP, such as adenosine monophosphate or adenosine, can induce the production of IL-10, an immunosuppressive cytokine, in a dose-dependent manner (Seo et al. 2004). They can also impair neutrophil chemotaxis and host defense during sepsis (Li et al. 2017). Therefore, ATP balance in the extracellular microenvironment is critical for the modulation of innate and adaptive immunity.

Immunosuppressive factors have been detected *in vivo* and *in vitro* in sera from patients with active tuberculosis, cirrhosis, and solid tumors (Sherwin et al. 1979; Sugiyama et al. 1987; O'Brien et al. 2014; Kano 2015). In sera from septic patients, common immunosuppressive factors have also been detected, including IL-10, transforming growth factor β, and IL-4; these cytokines suppress pro-inflammatory mediators, tissue repair, T cell proliferation, and differentiation, among other functions, but also accelerate fibrosis and the development of T regulatory cells (Schulte et al.

2013). Similarly, in our study, septic sera that were collected during early clinical phases were found to contain immunosuppressive factors including IL-10, which is known to repress cytokine production in lipopolysaccharide-stimulated peritoneal macrophages (Fiorentino et al. 1991). However, the role of IL-10 in ATP-induced IL-1β production is unknown, especially since neutralizing antibodies to IL-10 did not affect the response of THP-1 macrophages to extracellular ATP (data not shown). Thus, sera from septic patients likely contain suppressive factors independent of IL-10.

Our data also suggested that sera from septic patients who did not survive enhance $P2X_7$ uptake (Fig. 3D), which might partly explain the edema and cell swelling typically observed in septic patients. However, further studies are required to test this hypothesis. A lack of data regarding ATP trafficking is also a limitation of this study. For example, we observed that intracellular ATP levels were comparable upon stimulation with sera from septic patients who survived and those from non-survivors, probably as a result of autoregulatory mechanisms in the cytosol. Such mechanisms include the opening of pannexin pores to release ATP and IL-1β into the extracellular space, as well as feedback regulation by adenosine, which itself is regulated by membrane-bound nucleotide transporters and by intracellular adenosine kinase (Baron et al. 2015). Finally, the number of non-surviving patients was too small to investigate the relationship between a potential novel mechanism of immunoparalysis and mortality. Thus, we recommend larger multicenter studies, especially using non-survivors.

Conclusion

In conclusion, we show that immunoparalysis in septic patients could be caused by serum factor(s) that suppress the inflammatory IL-1β response to extracellular ATP by inhibiting caspase-1 activation in innate immune cells. Such suppressive factors might be correlated with fatal outcomes.

Abbreviations

ASC, apoptosis-associated speck-like protein; ATP, adenosine triphosphate; DAMP, damage-associated molecular patterns; HMGB1, high mobility group box 1; NLRP3, nucleotide oligomerization domain-like receptor leucine-rich repeat containing protein 3; PYCARD, N-terminal PYRIN-PAAD-DAPIN domain (PYD) and C-terminal caspase-recruitment domain (CARD); IL, interleukin; THP-1, human acute monocytic leukemia cells.

Acknowledgements

The authors thank doctors, nurses, technicians, and patients at the Department of Emergency and Critical Care Medicine, Hiroshima University Hospital, who participated in and enabled this study. The Office of Medical Science, Hiroshima University Hospital supervised the study, including study design, protocol, ethical issues, data collection, and case report forms.

Funding

Professor Nobuyuki Hirohashi and Ho Minh Van were funded through the Hiroshima University Phoenix Leader Education Program (Hiroshima Initiative), a part of the Renaissance from Radiation Disaster project by the Ministry of Education, Culture, Sports, Science and Technology, Japan. Professor Nobuaki Shime was funded by JSPS KAKENHI (Grant Number 17K11573). Professor Masamoto Kanno was funded by JSPS KAKENHI (Grant Number JP26500010), by CREST Agency for Medical Research and Development, Japan (Grant Number

17gm0710009h0204), and by SENTAN Agency for Medical Research and Development, Japan (Grant Number 17hm0102012h0005).

Author contributions

HMV participated in study design, data collection, drafting of the manuscript, and statistical analysis. KO, JI, SY, and KS helped collect sera and obtained and analyzed clinical data. WSK and GY helped with experiments and drafting of the manuscript. NH participated in study design, statistical analysis, and drafting of the manuscript. KT participated in study design and coordination. MK helped design the study and draft the manuscript. NS participated in the study conception and design, data collection, and drafting of the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interests

The authors declare that there are no conflicts of interest.

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Table 1. Baseline characteristics of cohorts.

Parameters	Controls (n = 5)	Trauma (n = 10)	Sepsis (n = 49)
Age, y	67 ± 6	68 ± 10	68 ± 14
Men	4 (80)	6 (60)	25 (51)
Sepsis/severe sepsis and septic shock	NA	NA	13/36
Injury severity score	NA	35 [25–42]	NA
Head and neck injury, region 1	NA	4 (40)	NA
Facial, region 2	NA	1 (10)	NA
Chest, region 3	NA	3 (30)	NA
Extremities, region 5	NA	2 (20)	NA
Site of original infection			
Abdomen	NA	NA	11 (28)
Central nervous system	NA	NA	1 (3)
Respiratory	NA	NA	5 (13)
Soft tissue	NA	NA	13 (33)
Urinary tract	NA	NA	10 (26)
Others	NA	NA	3 (8)
Undetermined	NA	NA	6 (15)
Serum concentrations, mg/L			
C-reactive protein	NA	0.19 ± 0.05	17.8 ± 11.0
Procalcitonin	NA	Not measured	8.7 [2.0–10.0]
Lactate	NA	1.9 ± 0.3	5.3 ± 4.9
Scores			
Acute Physiologic Assessment and Chronic Health Evaluation II	NA	4.6 ± 1.2	20.8 ± 8.5
Sequential Organ Failure Assessment Score	NA	5.3 ± 2.5	8.7 ± 4.7
Disseminated Intravascular Coagulation[□]			
Mean score	NA	3.0 ± 1.2	4.2 ± 2.1
Numbers of patients with disseminated intravascular coagulation	NA	3 (30)	27 (55)
Survivors	5 (100)	10 (100)	39 (80)
Days in intensive care unit	NA	6 [4–13]	12 [7–21]

Data are mean ± SD, median [IQR], or number of observations (%).

NA, not applicable.

[□]Disseminated Intravascular Coagulation was calculated based on JAAM-DIC 2016

5

Table 2. Baseline characteristics of septic patients.

Parameters	**Survivors (n = 39)**	**Non-survivors (n = 10)**	***P***
Age, y	66 ± 1	72 ± 10	
Women	19 (49)	5 (50)	
Sepsis classification at admission			
Uncomplicated sepsis	13 (33)	-	
Severe sepsis	6 (15)	1 (10)	
Septic shock	20 (52)	9 (90)	
Site of original infection			
Abdomen	7 (18)	4 (40)	
Central nervous system	1 (3)	-	
Respiratory	3 (8)	2 (20)	
Soft tissue	13 (33)	-	
Urinary tract	9 (22)	1 (10)	
Others	3 (8)	-	
Undetermined	3 (8)	3 (30)	
Serum concentrations, mg/L			
C-reactive protein	19.0 ± 11.2	13.4 ± 9.3	0.14
Procalcitonin, median [IQR]	6.8 [1.5–10.3]	27.8 [24.2–51.3]	< 0.001
Lactate	3.5 ± 2.2	12.0 ± 4.1	< 0.0001
Score			
Acute Physiologic Assessment and Chronic Health Evaluation II	18.8 ± 7.2	28.8 ± 10.2	< 0.0001
Sequential Organ Failure Assessment	7.6 ± 3.4	13.3 ± 5.7	< 0.0001
Disseminated Intravascular Coagulation[□]			
Mean ± SD	3.9 ± 1.2	5.6 ± 2.8	0.023
n (%)	18/21 (46)	9 (90)	
Days spent in ICU, no [IQR]	13 [7–21]	12 [5–18]	

Unless specified otherwise, values are mean ± SD or numbers of observations (%).

[□]Disseminated Intravascular Coagulation was calculated based on JAAM-DIC 2016.

5

Figure legends

Fig. 1. Concentration of cytokines and chemokines in sera from septic patients and control individuals.

Sera collected at the time of patient admission were screened by bead-based fluorescence assays to quantify inflammatory cytokines, with a limit of quantification of 0.6 pg/mL. Individual serum samples were assayed in duplicate and averaged (healthy controls, n = 5; trauma patients, n = 10; sepsis patients, n = 49). *$P < 0.05$; **$P < 0.01$ by Mann-Whitney U test.

Fig. 2. IL-1β secretion is suppressed in THP-1 macrophages exposed to septic sera.

A–D. IL-1β secretion after an 8-h exposure to sera diluted 60× and supplemented without damage-associated molecular pattern molecules (A) or with 2 mM ATP (B), 200 mg/mL monosodium urate (C), and 1 μg/mL HMGB1 (D). THP-1 macrophages were treated with RPMI supplemented without (untreated) or with the indicated agent and sera diluted 60×. Individual serum samples were assayed in triplicate and averaged (healthy controls, n = 5; trauma patients, n = 10; sepsis patients, n = 49). **$P < 0.01$ by Mann-Whitney U test.

Fig. 3. Septic sera specifically suppress ATP-induced IL-1β production in THP-1 macrophages.

A, B. IL-1β secretion after an 8-h exposure to sera collected on days 1, 3, and 7 from septic patients who did (open bars) or did not survive (filled bars), which was supplemented with 2 mM ATP (A) or 200 mg/mL monosodium urate (MSU) (B). Macrophages were treated with RPMI supplemented without (untreated) or with the indicated agent and sera diluted 60×. Individual sera were assayed in triplicate and averaged (sepsis survivors on day 1, n = 39; sepsis

non-survivors on day 1, n = 10; sepsis survivors on day 3, n = 16; sepsis non-survivors on day 3, n = 4; sepsis survivors on day 7, n = 16; sepsis non-survivors on day 7, n = 4). *$P < 0.05$, by χ^2 test.

Fig. 4. Sera from septic patients who did not survive activate $P2X_7$ uptake.

A–B. ATP in the culture supernatant was quantified by a luciferin-luciferase assay using the supernatant of macrophages exposed to ATP and sera for 8 h (A) and in sera collected at the time of patient admission (B). C. Cells treated with ATP and sera were also treated with YO-PRO-1 dye and analyzed by flow cytometry. D. Cells treated with ATP and sera from septic patients who did (clear bars) or did not survive (filled bars) were incubated with YO-PRO-1 dye and analyzed by flow cytometry. Macrophages were treated with RPMI supplemented without (untreated) or with the indicated agent and sera diluted 60×. Individual sera were tested in triplicate and averaged (healthy controls, n = 5; trauma patients, n = 10; sepsis patients on day 1, n = 49; sepsis patients on day 7, n = 20; sepsis survivors on day 1, n = 39; sepsis non-survivors on day 1, n = 10; sepsis survivors on day 7, n = 16; sepsis non-survivors on day 7, n = 4). *$P <$ 0.05 by Mann-Whitney U test; n.s., not significant by ANOVA.

Fig. 5. Suppression of intracellular signaling mediators upstream of NLRP3 by sera from septic patients.

Lysates from macrophages exposed to ATP and sera were assayed by ELISA for intracellular ATP (A), inositol 1,4,5-triphosphate (B), cytosolic calcium (C), and cyclic AMP (D). Macrophages were treated with RPMI supplemented without (untreated) or with the indicated

agent and sera diluted 60×. Individual sera were assayed in triplicate and averaged (healthy controls, n = 5; trauma patients, n = 10; sepsis patients on day 1, n = 49; sepsis patients on day 7, n = 20). *$P < 0.05$; **$P < 0.01$ by Mann-Whitney U test or χ^2 test.

Fig. 6. Sera from septic patients who did not survive suppress active caspase-1 in macrophages.

A, B. Pro-IL-1β (A) and activated caspase-1 (B) expression in cells treated with ATP and sera for 8 h. C. Production of active caspase-1 in cells treated with ATP and sera collected at the time of admission and on day 7 from septic patients who did (clear bars) or did not survive (filled bars). D. Production of intracellular IL-1β in cells treated for 8 h with ATP and sera. E. Production of intracellular IL-1β in cells treated for 8 h with ATP and sera collected at the time of patient admission and on day 7 from sepsis patients who did (clear bars) and did not survive (filled bars). Macrophages were treated with RPMI supplemented without (untreated) or with the indicated agent and sera diluted 60×. Individual serum samples were assayed in triplicate and averaged (healthy controls, n = 5; trauma patients, n = 10; sepsis patients on day 1, n = 49; sepsis patients on day 7, n = 20; sepsis survivors on day 1, n = 39; sepsis non-survivors on day 1, n = 10; sepsis survivors on day 7, n = 16; sepsis non-survivors on day 7, n = 4). *$P < 0.05$; **$P < 0.01$ by Mann-Whitney U test or χ^2 test.

Fig. 1

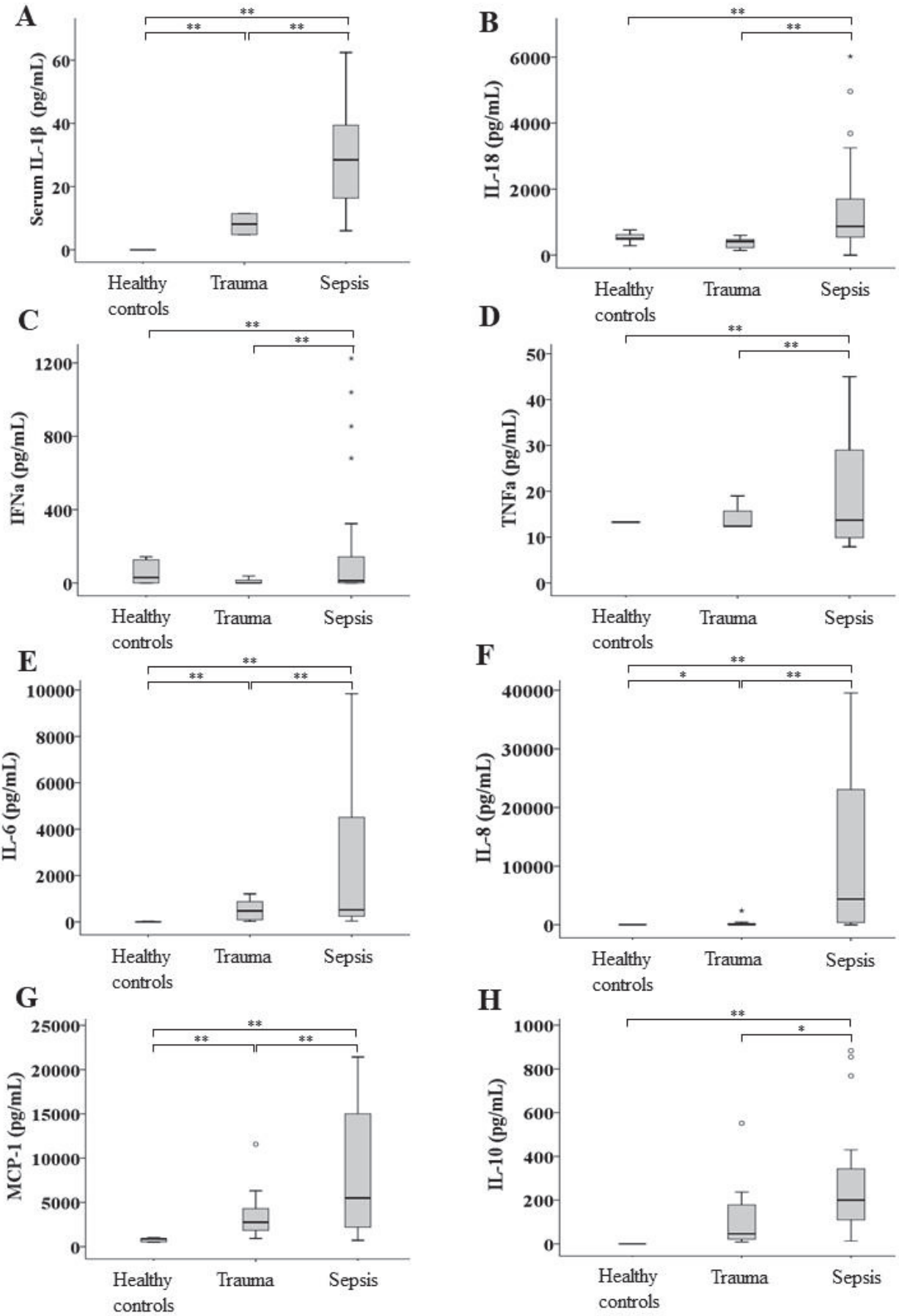

A
Serum IL-1β (pg/mL)
0
20
40
60
**
**
**
Healthy controls
Trauma
Sepsis
B
IL-18 (pg/mL)
0
2000
4000
6000
**
**
Healthy controls
Trauma
Sepsis
C
IFNa (pg/mL)
0
400
800
1200
**
**
Healthy controls
Trauma
Sepsis
D
TNFa (pg/mL)
0
10
20
30
40
50
**
**
Healthy controls
Trauma
Sepsis
E
IL-6 (pg/mL)
0
2000
4000
6000
8000
10000
**
**
**
Healthy controls
Trauma
Sepsis
F
IL-8 (pg/mL)
0
10000
20000
30000
40000
**
*
**
Healthy controls
Trauma
Sepsis
G
MCP-1 (pg/mL)
0
5000
10000
15000
20000
25000
**
**
**
Healthy controls
Trauma
Sepsis
H
IL-10 (pg/mL)
0
200
400
600
800
1000
**
*
Healthy controls
Trauma
Sepsis

Fig. 2

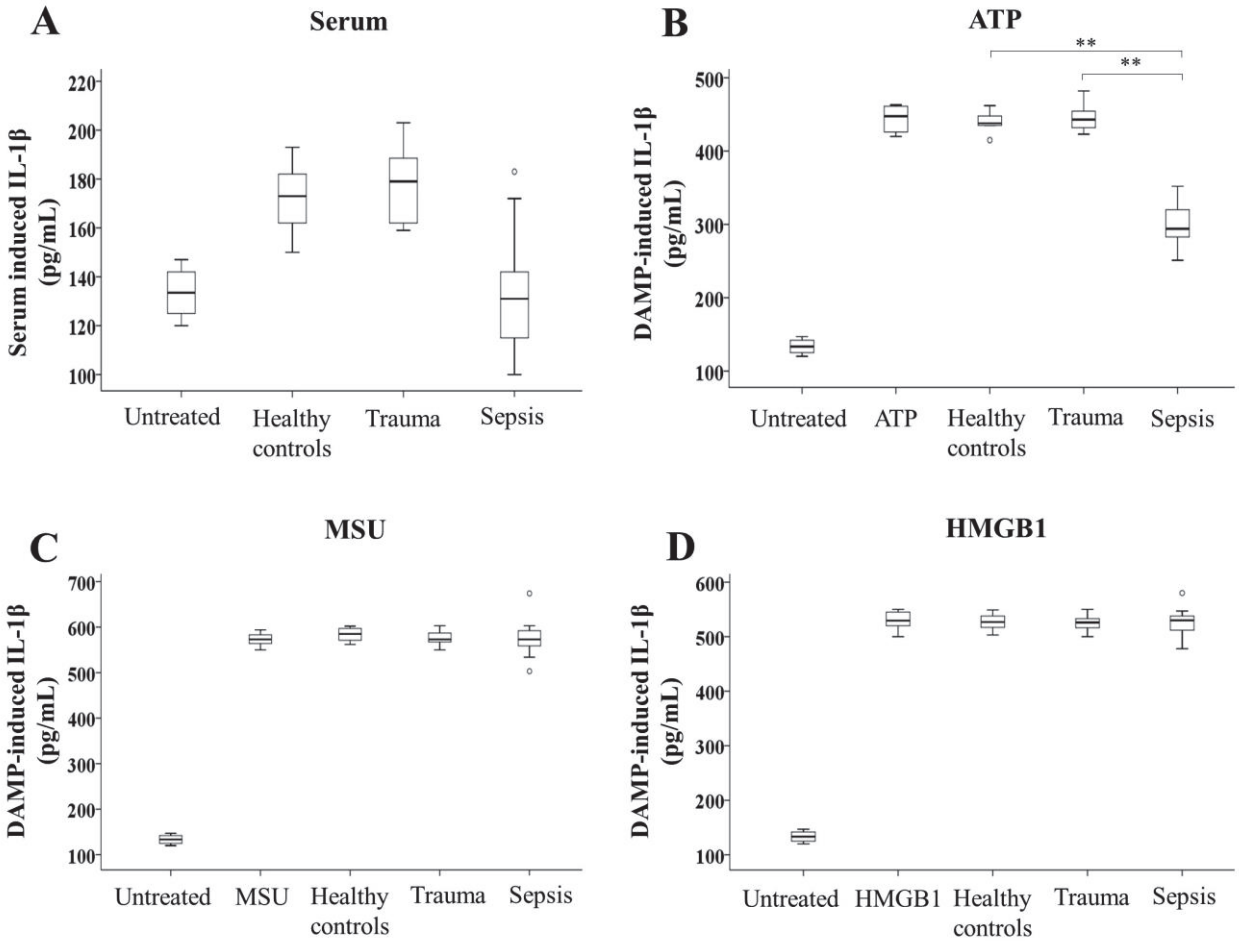
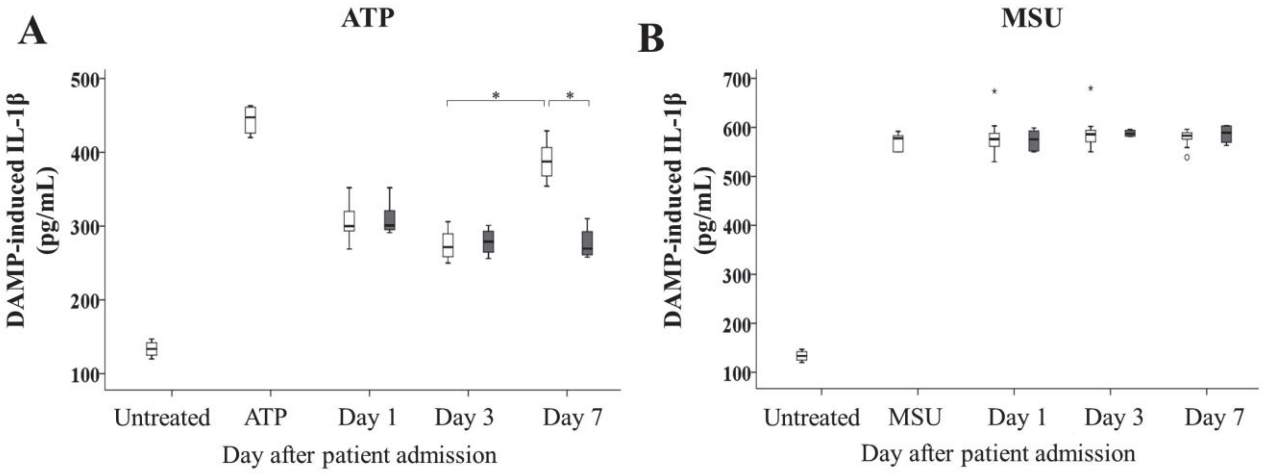

A
Serum
Serum induced IL-1β (pg/mL)
220
200
180
160
140
120
100
Untreated
Healthy controls
Trauma
Sepsis
B
ATP
**
**
DAMP-induced IL-1β (pg/mL)
500
400
300
200
100
Untreated
ATP
Healthy controls
Trauma
Sepsis
C
MSU
DAMP-induced IL-1β (pg/mL)
700
600
500
400
300
200
100
Untreated
MSU
Healthy controls
Trauma
Sepsis
D
HMGB1
DAMP-induced IL-1β (pg/mL)
600
500
400
300
200
100
Untreated
HMGB1
Healthy controls
Trauma
Sepsis

Fig. 3


A
ATP
DAMP-induced IL-1β (pg/mL)
500
400
300
200
100
*
*
Untreated
ATP
Day 1
Day 3
Day 7
Day after patient admission
B
MSU
DAMP-induced IL-1β (pg/mL)
700
600
500
400
300
200
100
Untreated
MSU
Day 1
Day 3
Day 7
Day after patient admission

5

Fig. 4

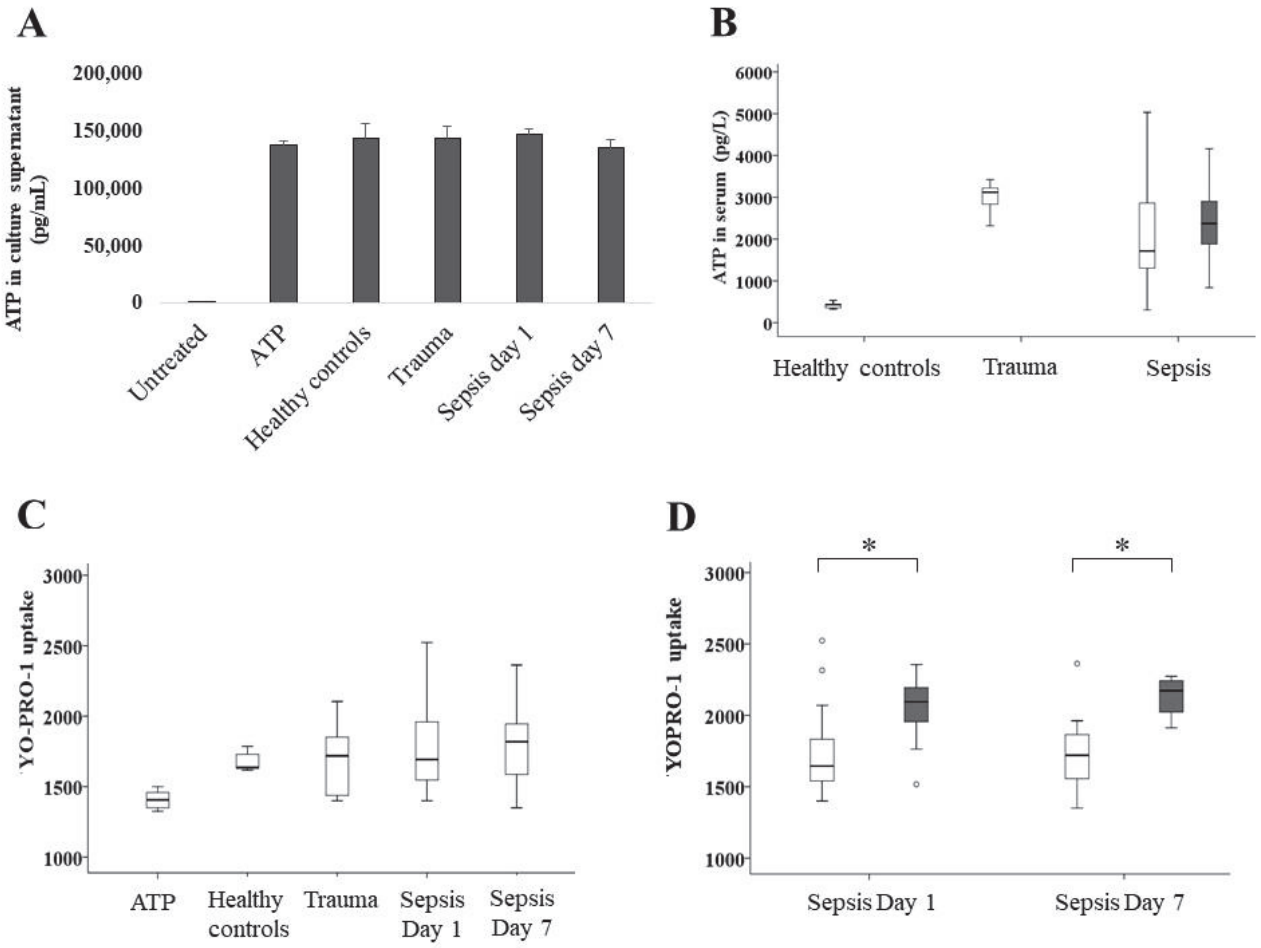

A
ATP in culture supernatant (pg/mL)
200,000
150,000
100,000
50,000
0
Untreated
ATP
Healthy controls
Trauma
Sepsis day 1
Sepsis day 7
B
ATP in serum (pg/L)
6000
5000
4000
3000
2000
1000
0
Healthy controls
Trauma
Sepsis
C
YO-PRO-1 uptake
3000
2500
2000
1500
1000
ATP
Healthy controls
Trauma
Sepsis Day 1
Sepsis Day 7
D
YOPRO-1 uptake
3000
2500
2000
1500
1000
*
*
Sepsis Day 1
Sepsis Day 7

Fig. 5

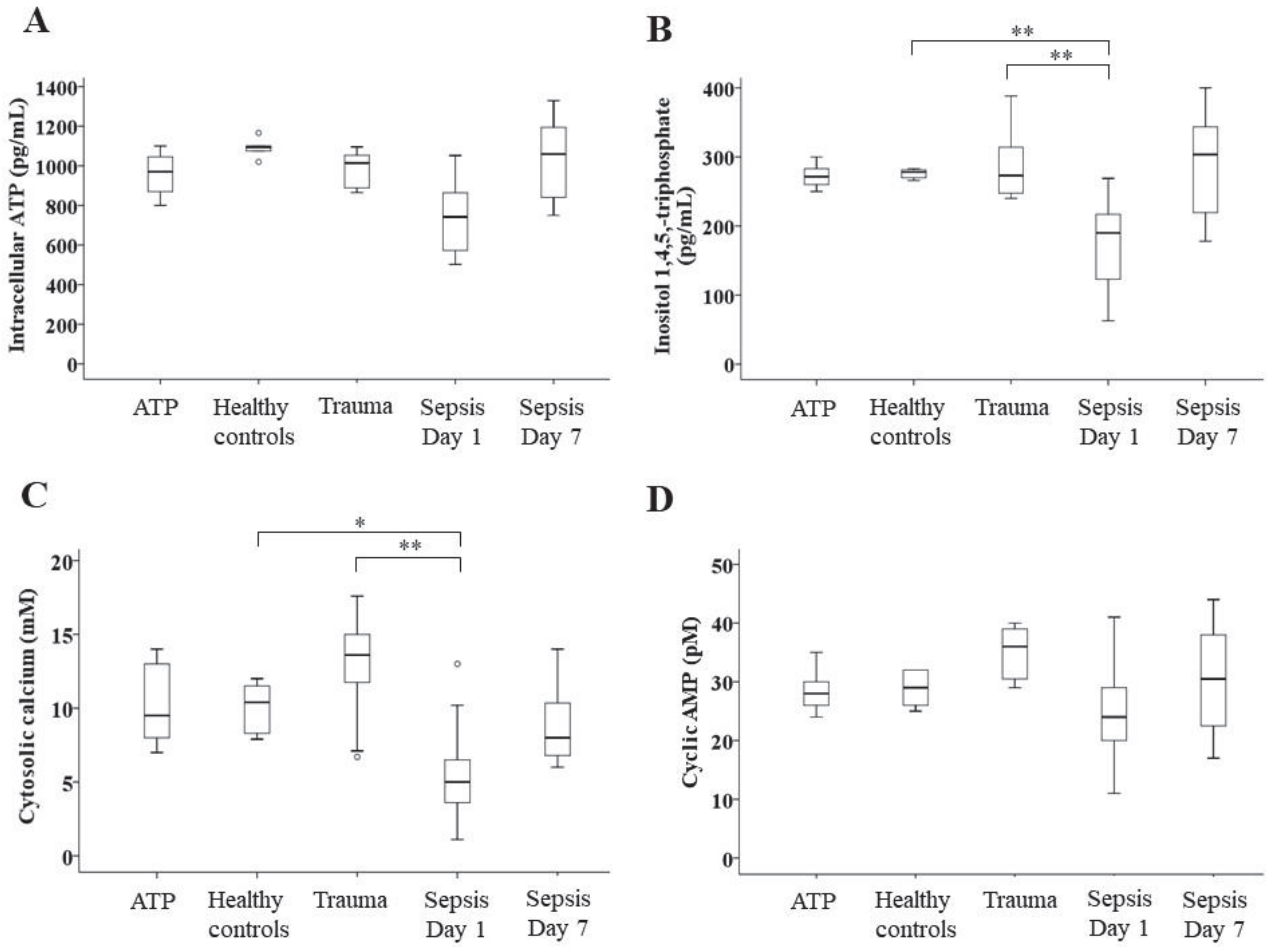
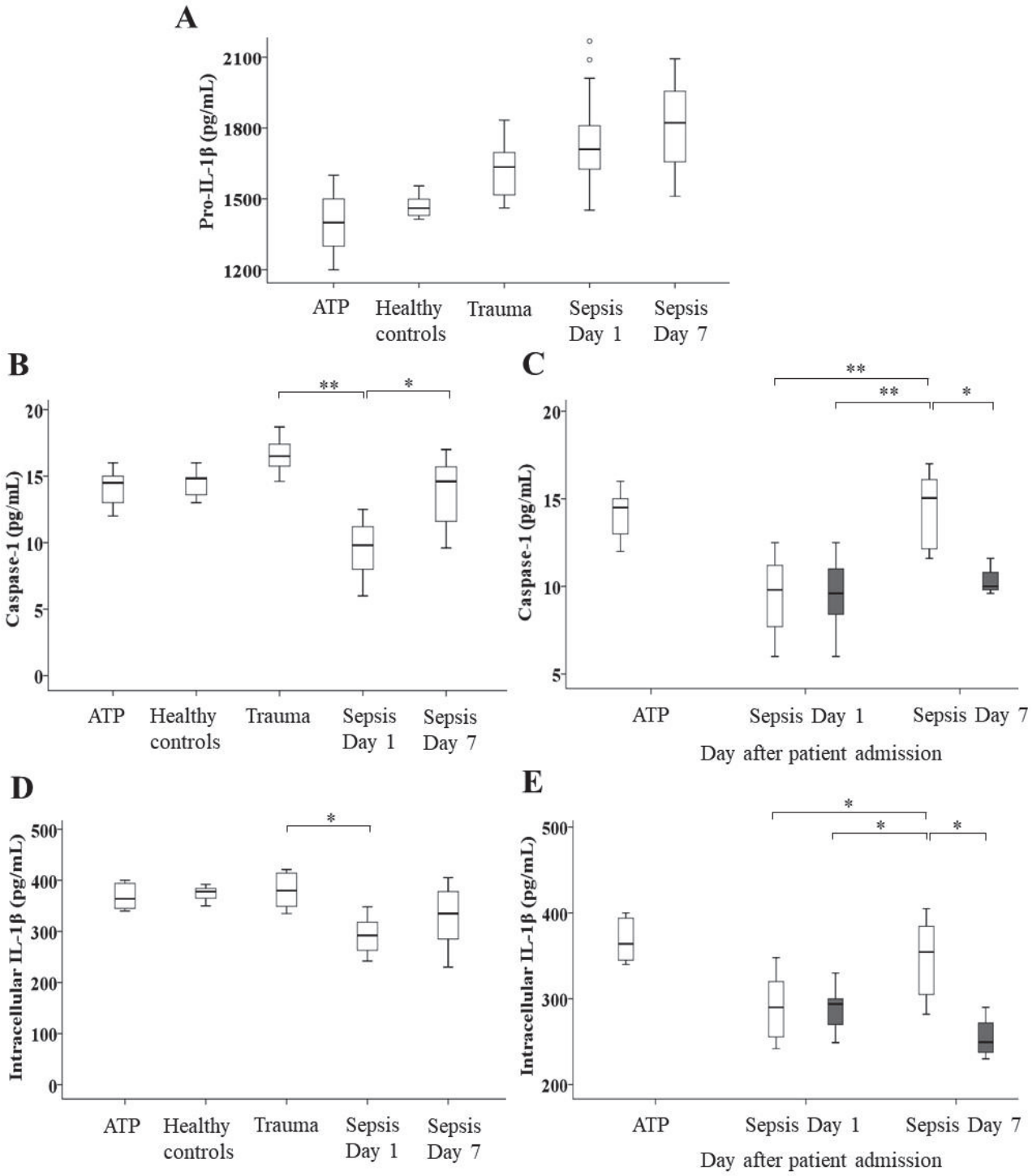

A
Intracellular ATP (pg/mL)
1400
1200
1000
800
600
400
200
0
ATP
Healthy controls
Trauma
Sepsis Day 1
Sepsis Day 7
B
Inositol 1,4,5,-triphosphate (pg/mL)
**
**
400
300
200
100
0
ATP
Healthy controls
Trauma
Sepsis Day 1
Sepsis Day 7
C
Cytosolic calcium (mM)
*
**
20
15
10
5
0
ATP
Healthy controls
Trauma
Sepsis Day 1
Sepsis Day 7
D
Cyclic AMP (pM)
50
40
30
20
10
0
ATP
Healthy controls
Trauma
Sepsis Day 1
Sepsis Day 7

Fig. 6


A
Pro-IL-1β (pg/mL)
2100
1800
1500
1200
ATP
Healthy controls
Trauma
Sepsis Day 1
Sepsis Day 7
B
Caspase-1 (pg/mL)
20
15
10
5
0
**
*
ATP
Healthy controls
Trauma
Sepsis Day 1
Sepsis Day 7
C
Caspase-1 (pg/mL)
20
15
10
5
**
**
*
ATP
Sepsis Day 1
Sepsis Day 7
Day after patient admission
D
Intracellular IL-1β (pg/mL)
500
400
300
200
100
0
*
ATP
Healthy controls
Trauma
Sepsis Day 1
Sepsis Day 7
E
Intracellular IL-1β (pg/mL)
500
400
300
200
*
*
*
ATP
Sepsis Day 1
Sepsis Day 7
Day after patient admission

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