Sustained Down Regulation of Plasma Tetranectin Contributes to Liver Injury in Pneumonia-Associated Sepsis

Shuaiwei Wang
Huaihe Hospital of Henan University
Yu Bai
Huaihe Hospital of Henan University
Yu Guo
Henan University
Qing Bai
Henan University
Xiangyan Wei
Henan University
Yijie Zhang
Henan University
Na Chen
Huaihe Hospital
Edmund J. Miller
RDS2 Solutions
Wei Li (10220038@vip.henu.edu.cn)
Henan University, Kaifeng, Henan CN https://orcid.org/0000-0002-7941-7022

Research

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Abstract

Background: It was recently shown that tetranectin (TN) concentration in the plasma of sepsis patients was significantly lower than healthy control and that exogenous TN reduced the mortality rate in septic mice. The aim of this study is to determine whether the reduction of plasma TN is a sepsis-specific host response and its impact on organ dysfunction in sepsis.

Methods: The study was conducted in the Sepsis Laboratory at the Huaihe Hospital of Henan University in China. Thirty-seven healthy, 30 community-acquired pneumonia (CAP) and 363 sepsis with comorbid pneumonia (SWP) subjects were recruited. A murine model of polymicrobial sepsis was used to characterize the role of plasma TN in sepsis pathogenesis.

Results: TN concentrations in plasma from both CAP and SWP subjects were lower than healthy controls, but not significantly different between male and female sepsis patients, before and after the occurrence or the resolution of sepsis. In addition, plasma TN was not associated with the occurrence of septic shock or sepsis mortality in both genders. On the other hand, plasma TN negatively correlated with liver injury indicators in moribund SWP subjects. In mice of polymicrobial sepsis, a significant decrease in plasma TN occurred within hours after the ligation and puncture of the cecum. Recombinant human TN induced significant reductions of tissue injury markers of liver, but not other organs. In addition, exogenous TN selectively reduced the level of receptor-interacting protein kinase 3 among a panel of cell death markers in septic mouse liver.

Conclusions: The dramatic and persistent down-regulation of plasma TN is not a sepsis-specific host response, but contributes to liver injury in pneumonia-associated sepsis.

Background

Sepsis is a life-threatening organ dysfunction caused by dysregulated host responses to infection and is a major cause of human death (1–3). The nature of organ dysfunction-induced host responses has not been adequately defined. A growing body of evidence, especially the failure of virtually all inflammation-targeting clinical trials, suggests that inflammation may not be the direct cause of sepsis-associated organ dysfunctions, but may trigger sustained organ-damaging responses that are not sensitive to anti-inflammation interventions (3–10). Characterization of organ-specific injurious host responses may aid in the development of sepsis therapies.

Sepsis patients often develop dysfunction in various organs, regardless of the location of initial infection (2, 3). The heterogeneity of impacted organs contributes to the prevalent occurrence of multiple organ dysfunctions especially in moribund sepsis patients, and suggests inter-organ propagation of injurious factors likely through the circulation (1, 3, 7, 10). The cause for the heterogeneity in impacted organs is poorly understood, but likely attributable to an imbalance of organ-specific protective and injurious protective factors.

Tetranectin (TN) is a 22 kDa protein encoded by the CLEC3B gene and was initially identified as a plasminogen-binding protein in human plasma (11). It was recently reported that the concentration of TN is significantly decreased in the serum of sepsis patients and septic mice, as compared with healthy controls (12). In addition, supplementation of recombinant human or mouse TN reduced the mortality rate of mice of polymicrobial sepsis (12). It is, however, not clear whether the reduction of TN concentration in septic plasma is a sepsis- or organ-specific host response.

In this study, we determined plasma concentrations of TN in healthy controls, and subjects with community-acquired pneumonia (CAP), sepsis developed from pneumonia (PtoS), pneumonia after sepsis resolution (StoP) or sepsis with comorbid pneumonia (SWP), and correlated plasma concentrations of TN and values of a panel of clinical tests, reflecting the status of inflammation, and the function of most critical organ systems. In addition, we assessed the role of TN in organ injury using a rodent model of polymicrobial sepsis.

Methods

Detailed information of materials is summarized in S-Table 1.

Inclusion and exclusion criteria of human subjects

Subjects of both genders between 50 and 85 years of age were randomly qualified initially, and subsequently screened with the following exclusion criteria: pregnancy, recent chemotherapy, use of immunosuppressants or steroid, and HIV or HBV positive.

Thirty-seven subjects with no existing medical conditions were enrolled as healthy controls. Thirty CAP subjects were diagnosed according to a recently updated guideline (13). Three hundred and sixty-three SWP subjects fulfilled the Sepsis 3.0 (1) diagnosis criteria of sepsis as well as pneumonia. PtoS refers to CAP subjects that were later qualified as SWP due to development of organ dysfunction, as determined by sequential [sepsis-related] organ failure assessment (SOFA), whereas StoP subjects were SWP patients that subsequently recovered from sepsis (SOFA $\leq$ 1), but retained symptoms of pneumonia. Sepsis-associated pneumonia (SAP) is a combination of PtoS and StoP groups.

Comorbidities of SWP patients was based on diagnosis on discharge, which reflected assessments of the course of disease progression during hospitalization. Values of a panel of clinical tests (S-Table 2) were obtained from hospital record. The mortality was determined by hospital record or follow-up after discharge.

Human blood collection and plasma preparation

Plasma preparation was conducted according to a protocol as previously described (14). Briefly, blood samples were obtained on the day of diagnosis of pneumonia or sepsis, centrifuged at 2,000g at 4 ^{o}C for

10 min within 2 hr after collection, and the resultant plasma was harvested, aliquoted and then kept at -80^{o}C until use.

Mouse model of polymicrobial sepsis

A total of 61 Balb/c male and 20 female mice (9-10 wks old, 23-27g) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). A mouse model of polymicrobial sepsis was generated by the ligation and puncture of cecum (CLP), as previously described (15, 16). For the characterization of changes in plasma TN in sepsis, CLP mice of both genders were allowed to survive 0, 6, 12, 24 and 48 hr before euthanization and subsequent intracardial blood collection. For the assessment of tissue injury, mice were allowed to survival to 6 and 18 hr post CLP. For the 6-hr survival group, mice received a single intravenous injection (via tail vein) either saline (+CLP+Saline) or recombinant TN (2 mg/kg, +CLP+TN) at 2 hr before euthanization. For the 18-hr survival group, mice were injected with either saline or TN once at 4 hr and once at 16 hr post CLP (2 hr before euthanization). Blood and tissue samples were collected at the end of survival time.

Western blotting

Western blotting was used to determine the relative levels of TN, high mobility group box 1 (HMGB1), alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), creatine kinase M (CKM), fibrinogen and its degradation products in mouse plasma or the level of cell injury markers such as caspase-1, -8 and -11, glutathione peroxidase 4 (GPX4) or receptor-interacting protein kinase 3 (RIP3) in livers from septic mice, as previously described (16).

TN ELISA

The concentration of TN in human plasma was determined using a human TN ELISA kit from Sino Biological (Beijing, China), according to a protocol provided by the manufacturer. One sample from each subject in healthy, CAP and SAP groups, and 1,132 samples from SWP groups (including multiple samples from 222 SWP subjects and single sample from the other 141 SWP subjects) were analyzed.

Direct bilirubin (DBIL), creatinine and blood urea nitrogen (BUN) assays

The levels of DBIL, creatinine and BUN in septic mouse plasma were determined using commercial test kits according to manufacturer's instructions.

Statistical analysis

Shapiro-Wilk normality test was performed to determine the distribution normality of variables. Variables with a normal distribution were analyzed using Student's t-test for comparison of two groups, or one-way ANOVA followed by Tukey test for multiple groups, and presented as mean $\pm$ standard deviation; Variables with a nonparametric distribution were analyzed using the Mann-Whitney U test for two groups, or the Kruskal-Wallis ANOVA test followed by the Dunn's test for multiple groups, and presented as

median and interquartile ranges. Spearman's rank-order correlation (r_s) analysis was performed to determine the correlation between plasma TN concentrations and values of clinical tests (S-Table 2). The statistical significance level was set at $p < 0.05$.

Results

Patients characteristics

The demographics and basic clinical characteristics of human subjects are shown in Table 1. The median age of healthy individuals was lower than SAP and SWP subjects, but not significantly different from the CAP group. No significant difference in age between CAP, SAP and SWP groups. The gender distribution was not significantly different between all groups.

Among 290 SWP patients that had the same day test of mean arterial blood pressure and lactate, 37 of 200 male (18.5%) and 18 of 90 female (20%) patients developed septic shock. The prognosis of 304 SWP patients was verified by hospital records or follow-up. The 30-day mortality rate were 61.19% (134 of 219) for male and 64.71 (55 of 85) for female patients. As shown in Table 1, the average SOFA score for SWP patients was 7.73 at the time of sepsis diagnosis. Among all six organ systems included in sepsis diagnosis criteria (1), dysfunction score of the respiratory system (SOFA_lung) ranked the highest. Accordant to the inclusion criteria, all SWP subjects had comorbidity of the respiratory system, followed by hypoproteinemia (55%) and gastrointestinal disorders (47%).

Plasma TN concentrations in healthy, pneumonia and sepsis subjects

By definition, infection is the necessary prelude for sepsis. In order to determine whether plasma TN is associated with sepsis pathogenesis, we compared the concentrations of TN in one plasma sample from each of plasma from 37 healthy, 30 CAP, 60 SAP and 148 SWP subjects, and multiple samples from 222 SWP subjects (1090 SWP samples in totality), using a human TN ELISA kit. The value of the first samples (collected on the day of diagnosis) of all SWP subject was used as representative of the SWP group. Consistent with the reported range (12, 17-19), the average TN concentration in healthy individuals was 11.27 $\pm$ 4.39 mg/L, the median concentration, however, was 2.12 mg/L (0.81-4.41, Fig. 1A). Compared with the healthy subjects, TN levels in CAP, SAP and sepsis plasma were 1.12 (0.76-1.40), 0.65 (0.32-1.61) and 0.92 mg/L (0.47-1.73), respectively (Fig. 1A), suggesting that plasma TN levels were reduced by 47.48%, 69.22% and 57% in CAP, SAP and SWP subjects, respectively.

As shown in Table 2, the plasma TN concentration was generally higher in female than male patients with various comorbidities, but the difference was not statistically significant in all comorbidity groups except hypoproteinemia ($p = 0.03$). No significant differences were found in TN concentrations before (0.51 mg/L 0.42-1.44) and after (0.65 mg/L, 0-2.86) the onset of sepsis in the PtoS group ($n = 11$, $p = 0.70$, Fig. 1B), or before (0.68 mg/L, 0.70-2.86) and after (0.84 mg/L, 0.84-1.67) sepsis resolution in the StoP group ($n = 55$, $p = 0.33$, Fig. 1C). Moreover, no significant correlations were found between TN

concentrations and the probability of occurrence of septic shock or 30 mortality rates of both genders, regardless of comorbidities.

The association of plasma TN with organ dysfunction in sepsis patients Correlation analysis showed that TN concentrations in SWP plasma had the highest, but nevertheless moderate, correlation with fibrinogen among values of 42 clinical tests (r_s = -0.29, Table 3). However, analysis of paired plasma samples (moderate and severe) from 31 moribund SWP subjects revealed a stronger and negative correlation between TN and liver dysfunction markers (Table 4), such as direct bilirubin (DBIL, r_s = -0.46) and total bilirubin (r_s = -0.42), indicating an inverse relation between plasma TN and liver injury in the course of sepsis-induced mortality.

The impact of recombinant TN on tissue injury in septic mice

To investigate the role of plasma TN in sepsis, we first determined whether the induction of sepsis is accompanied by a down regulation of plasma TN in septic mice (Fig. 2). As expected, a significant reduction of plasma TN occurred at 6 hr post CLP, which persisted in the following 42 hr, in both genders (Fig. 2A, B), demonstrating that the CLP-induced peritoneal infection caused a rapid and massive down regulation of plasma TN in a gender-independent manner.

As shown in Fig. 3, the concentrations of tissue injury markers, such as ALT (3A), DBIL (3B), AST (3C), LDH (3D) and CKM (3E), but not creatinine (3F), in mouse plasma exhibited significant elevations at 18 hr after the induction of sepsis. Intravenous administration of rHuTN at the dose of 2 mg/kg, which is equivalent to median TN concentration in normal human plasma, resulted in 28.43% and 42.56% decreases in liver-specific injury markers, ALT (p = 0.009) and DBIL (p = 0.03), respectively. In contrast to these liver injury markers, TN did not have any significant effects on non-selective tissue injury markers, such as AST and LDH, or markers for cardiac (CKM) and kidney (creatinine) injuries in septic mouse plasma. These results suggest that recombinant TN is protective of the liver in septic mice, corresponding to the correlation of plasma TN and the functional state of liver in sepsis patients.

Septic liver injury is associated with an activation of multiple mechanisms of cell death (20-22). As shown in Figure 4, we examined the levels of a panel of cell death markers in normal (-LPS) and sepsis mice. As shown in Figure 4, sepsis mouse liver (+CLP+Saline) contained significantly higher levels of RIP3, caspase-11 and GPX4, and lower levels of caspase-8, but no significant difference in caspase-1 at 18 after CLP, as compared with normal mice (-LPS). Intravenous administration of rHuTN (+LPS+TN) significantly reduced the level of RIP3 (p = 0.04), but not the others.

Discussion

By determining the plasma TN concentration of healthy, pneumonia and sepsis subjects and characterizing the correlation of plasma TN level and values of a variety of clinical parameters in sepsis patients, we found that both pneumonia in human and peritonitis in mouse can cause a dramatic down regulation of plasma TN. The reduction of TN concentration is likely not a sepsis-specific event, and is

not related to the occurrence or resolution of sepsis, but may contributes to the liver injury and dysfunction in moribund sepsis patients.

TN was initially identified as a plasminogen-binding protein in human plasma (11). A decrease in plasma TN has been observed in inflammatory diseases, such as ischemia, trauma and rheumatoid arthritis (17–19). Recently, Chen et al. found that administration of endotoxin or induction of peritonitis by CLP can cause a rapid decrease in plasma TN in mice (12). Our animal studies confirmed the finding by Chen et al. and, together, suggest that TN is a negative acute phase protein in a variety of infectious and non-infectious inflammatory diseases.

Compared with healthy subjects, plasma TN levels remained depressed in CAP, SAP and SWP subjects. Importantly, there was no significant change in the concentration of plasma TN before and after the development of sepsis in PtoS subjects, or the resolution of sepsis in StoP subjects. Moreover, the plasma TN level was not significantly associated with the occurrence of septic shock or 30-day mortality in both genders, regardless of comorbidities. These results suggest, apparently, that plasma TN is not a critical factor in the development, progression or prognosis of sepsis, at least in patients with comorbid pneumonia.

In contrast to the insignificant role of plasma TN in sepsis patients, Chen et al. (12) showed in a mouse model of sepsis that a deficiency in TN expression was associated with increased mortality, whereas administrations of recombinant TN or TN domain-specific monoclonal antibodies resulted in higher survival rates, suggesting an important role of TN in the (prevention of) pathogenesis of sepsis, at least in rodents.

The cause for this discrepancy is unknown. In SWP patients, the average dysfunction score of liver (0.53) was the lowest among all six organs (0.53–2.71, Table 1), indicating an overall very mild level of liver dysfunction. As a result, the association between plasma TN with liver dysfunction may have only very limited, and consequently insignificant, impact on sepsis mortality in SWP patients. In septic mice, however, liver injury appears to be a prominent pathological component, as indicated by over 4- and 13-fold increases in the plasma level of ALT and DBIL over control mice (Fig. 3). In fact, hepatocyte-specific deficiency in the expression of a damage-associated molecular pattern, high mobility groups box-1, resulted in a marked reduction of the mortality rate of CLP mice (23). Thus, the extent of liver injury may have a substantial impact on the outcome in septic mice. Consequently, the apparently preferential liver protection by recombinant TN in septic mice may manifest in improved survival, as previously reported (12).

Sepsis-associated liver injury can be caused by activation of various mechanisms of cell death (20–22). Among a panel of cell injury markers, CLP induced most dramatic increases of RIP3 (26-fold) and caspase-11 (5-fold), a significant decrease of caspase-8, but did not have any significant impacts on caspase-1 and GPX4, in septic mouse liver. Administration of TN only caused significant reduction of RIP3, but not caspase-1, caspase-11, GPX4 and caspase-8, suggesting that the liver protective effect of TN is mediated, at least in part, through an inhibition of RIP3-mediated cell death.

The selective liver protection suggests a unique targeting mechanism of TN, which remains to be investigated due to a lack of understanding of TN receptors. Alternatively, a number of TN-binding factors have been identified in plasma, including plasminogen, fibrin, heparin hepatic growth factor and lipoprotein(a) (11, 24–27). Hepatic receptors for TN, or its binding partners, deserve particular attention in the clarification of the role of TN in sepsis.

Conclusion

In this study, we demonstrated that plasma TN is a negative APP. The dramatic and sustained down regulation of plasma TN is a respiratory infection-induced and sepsis-independent host response that may contribute to sepsis-associated liver injury, possibly by compromising the control of RIP3-mediated cell death. Supplementation of exogenous TN may hold therapeutic potential for the prevention or treatment of liver dysfunction in sepsis.

Abbreviations

ALT: alanine aminotransferase

AST: aspartate aminotransferase

CAP: community-acquired pneumonia

CKM: creatine kinase M

CLP: cecal ligation and puncture

DBIL: direct bilirubin

GPX4: glutathione peroxidase 4

LDH: lactate dehydrogenase

PtoS: Pneumonia progressing to SWP

rHuTN: recombinant human tetranectin

RIP3: receptor-interacting protein kinase 3

SAP: sepsis-associated pneumonia

SOFA: sequential [sepsis-related] organ failure assessment

StoP: Pneumonia after resolution of SWP

SWP: sepsis with comorbid pneumonia

TN: tetranectin

Declarations

Ethics approval and consent to participate

The research protocol involving human subjects has been approved by the Medical Ethical Committee of Henan University, and informed consent was obtained prior to a subject's enrollment into the study. Laboratory animals were used in accordance with the guidelines of the Animal Care and Use Committee of the Huaihe Hospital of the Henan University.

Consent for publication

All authors have read the manuscript and agree to the submission for publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author (WL) or reasonable request.

Competing interests

Author Wei Li is an inventor in a related patent application in the USA. The remaining authors have disclosed that they do not have any conflicts of interest.

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Authors' contributions

SW, YB, YG, QB and XW performed the experiments, YZ, NC and WL analyzed the data, EJM and WL wrote the manuscript.

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Tables

Due to technical limitations, table 1,2,3,4 is only available as a download in the Supplemental Files section.

Figures

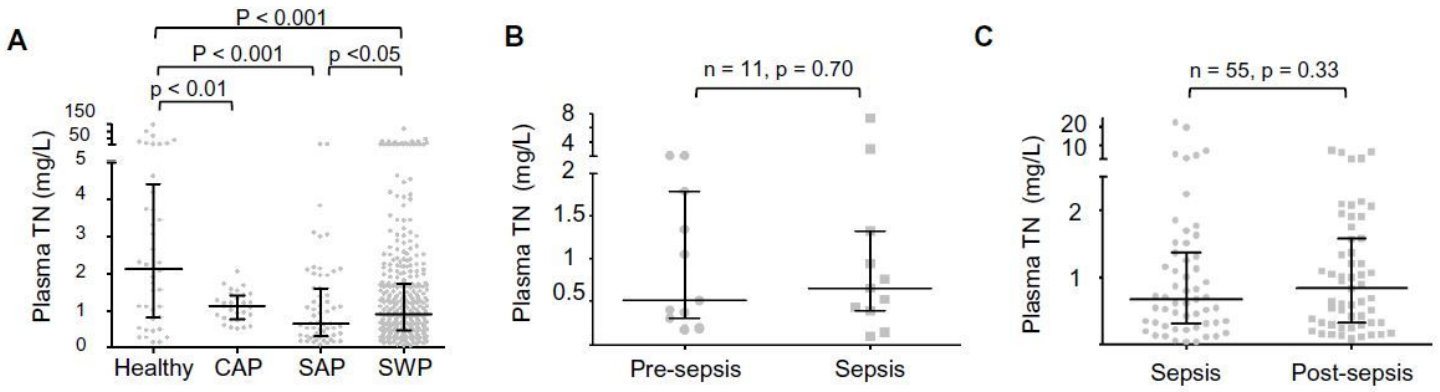

Figure 1

Plasma tetranectin (TN) concentrations in healthy, pneumonia and sepsis subjects. A. TN ELISA analysis of plasma from healthy (n = 37), community-acquired pneumonia (CAP, n = 30), sepsis-associated pneumonia (SAP, n = 60) and sepsis with comorbid pneumonia (SWP, n = 363) subjects. B. TN concentrations in paired plasma from 11 PtoS subjects before and after developing from pneumonia (Pre-sepsis) to sepsis (Sepsis). C. TN concentrations in 55 paired plasma from StoP subjects before (Sepsis) and after sepsis resolution (Post-sepsis). The Kruskal-Wallis ANOVA test followed by the Dunn's test was used in A; paired t-test was used in B and C; all values are shown as medians and interquartile ranges.

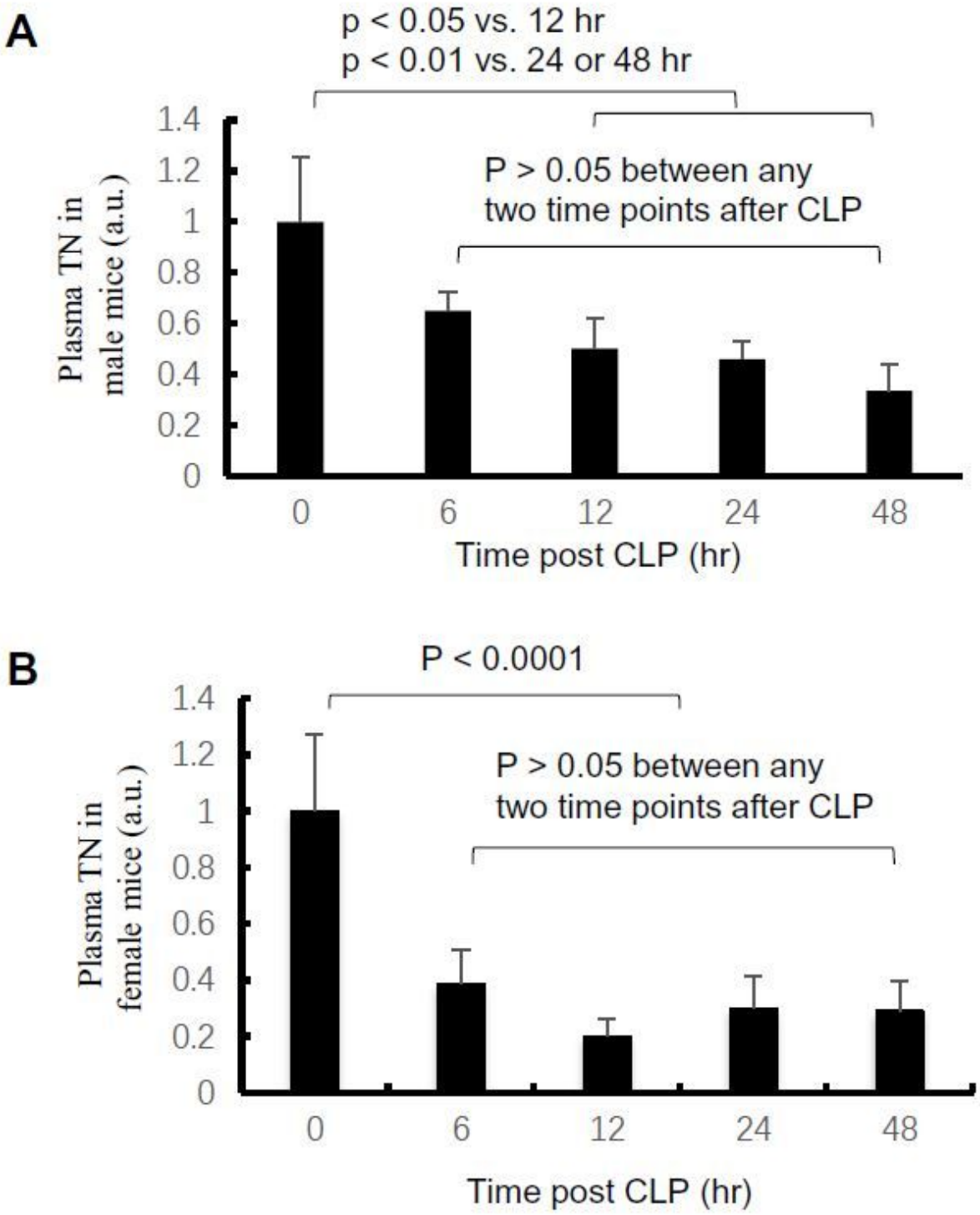

Figure 2

Reduction of plasma tetranectin (TN) levels in male (A) and female (B) septic mice. Plasma samples were obtained at designated time after the onset of sepsis (CLP). An equal volume (0.25 μl) of each sample was resolved by SDS-PAGE, and detected with a polyclonal anti-TN antibody. TN protein signal was quantified using the ImageJ software, and analyzed one-way ANOVA followed by Tukey test (two-tailed). a.u.: arbitrary unit; n = 3 in A; n = 4 in B; values are mean and standard deviations.

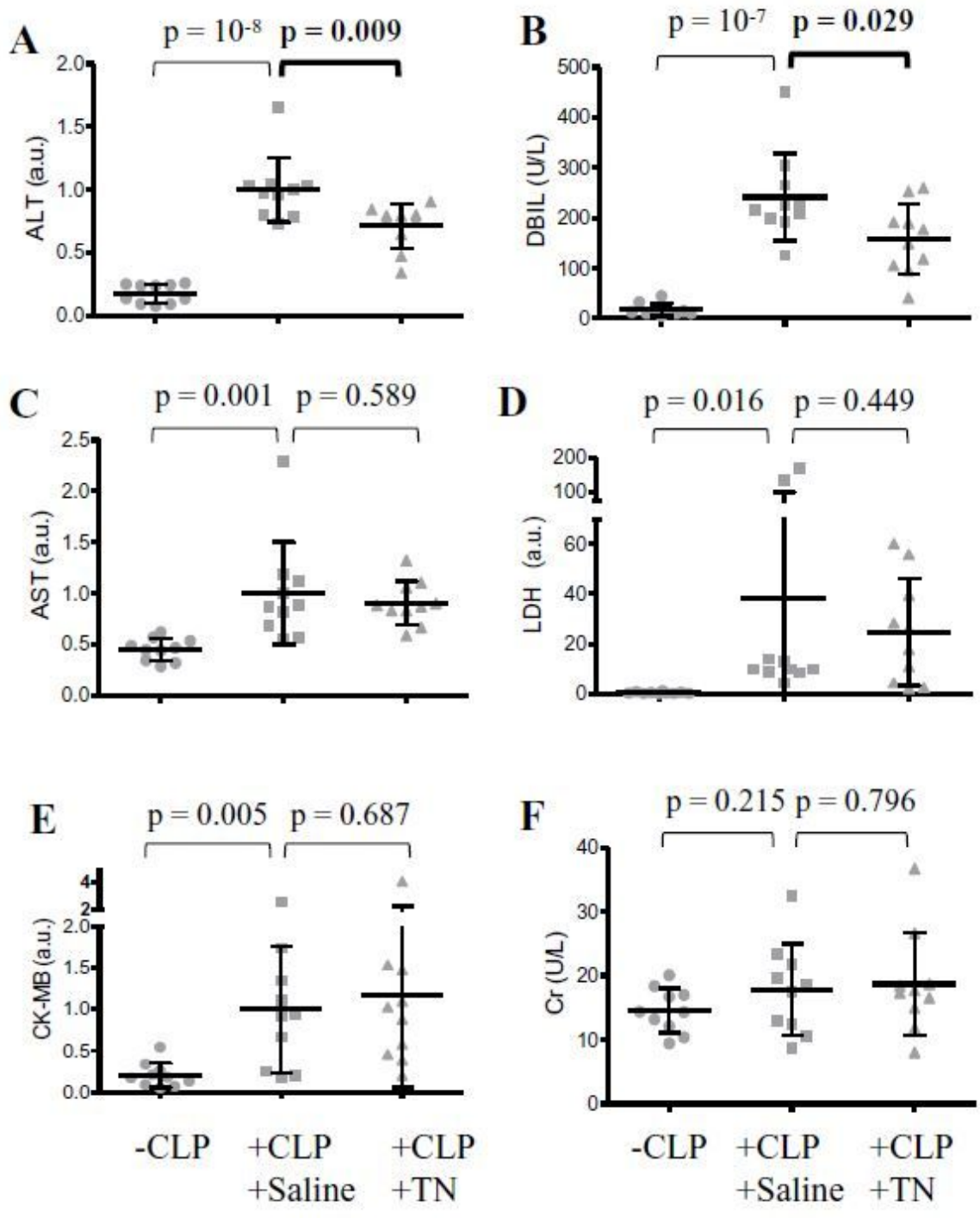

Figure 3

Effects of recombinant human tetranectin (rHuTN) on tissue injury markers in the plasma of septic mice. rHuTN (2.0 mg/kg) was administered (iv) once at 2 hr before euthanization (i.e. 4 hr post cecal ligation and puncture) for the 6 hr survival group and twice at 4 and 16 hr post CLP (i.e. 2 hr before euthanization) for the 18 hr survival group. Blood was drawn at the end of designated survival time for plasma preparation. Equal volume of plasma (0.1 µl) was resolved by SDS-PAGE for Western blotting of alanine

aminotransferase (ALT, A), aspartate aminotransferase (AST, C), creatine kinase M (CKM, E) and lactate dehydrogenase (LDH, D). Direct bilirubin (DBIL, B) and creatinine (Cr, F) were determined with 7 μl plasma from each mouse. One-way ANOVA followed by Tukey test was used for two-tailed group comparisons. Values are mean and standard deviations. n = 10;

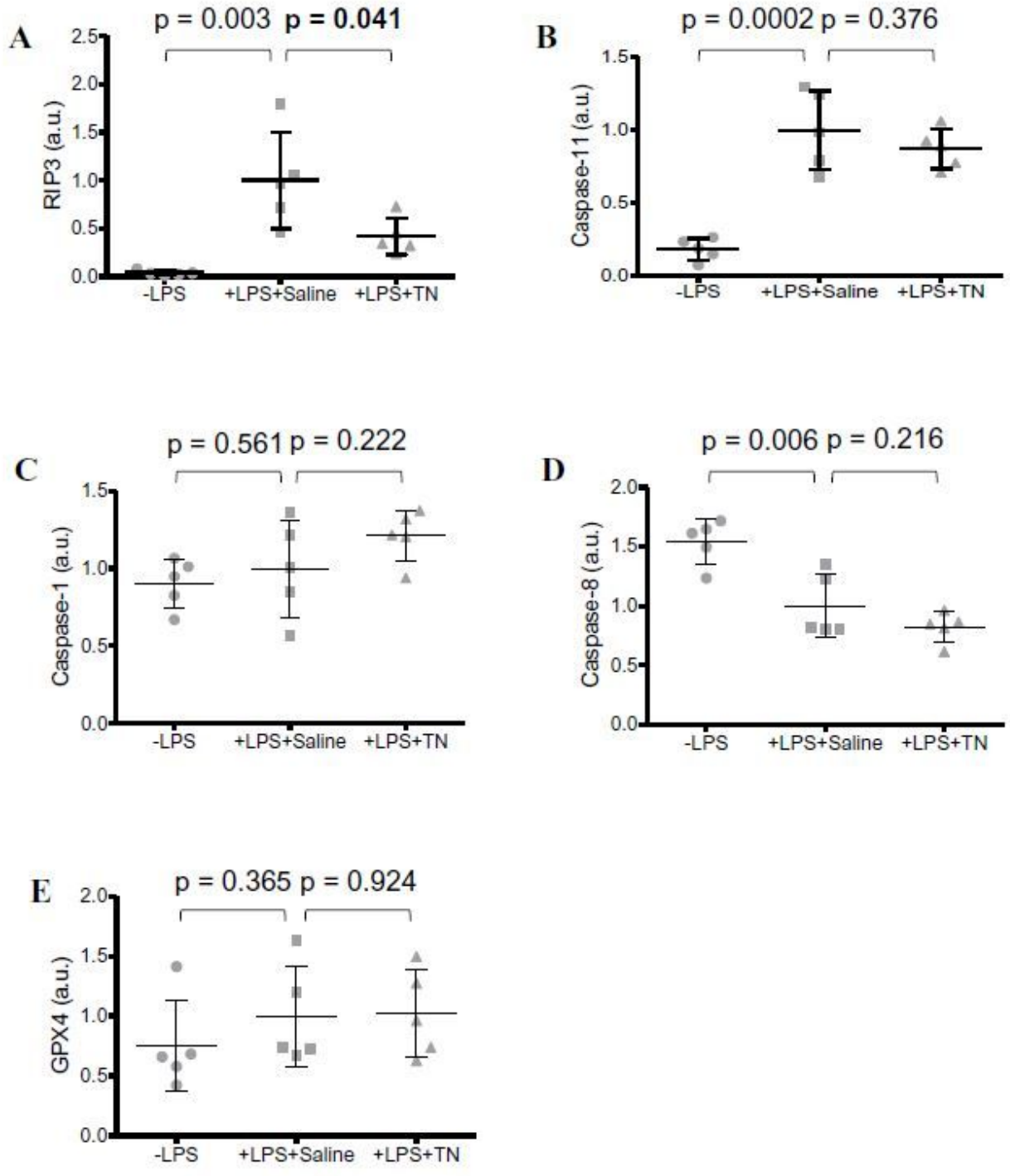

Figure 4

Effects of recombinant human tetranectin (rHuTN) on cell death markers in septic mouse liver. rHuTN (2.0 mg/kg) was administered (iv) twice at 4 and 16 hr post CLP (2 hr before euthanization) for the 18 hr survival group. Liver samples from normal or CLP mice were harvested after transcardial perfusion with PBS, and homogenized for SDS-PAGE (20 μg protein/sample). Western blotting of receptor-interacting protein kinase 3 (RIP3, A), caspase-1 (Cas-1, C), -8 (Cas-8, D) and -11 (Cas-11, B), and glutathione peroxidase 4 (GPX4, E) was performed. One-way ANOVA followed by Tukey test was used for two-tailed group comparisons. Values are mean and standard deviations. n = 5.

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