# Tansmembrane TNF-α as a novel biomarker for the diagnosis of cytokine storm in a mouse model of multiple organ failure

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**Research Article**

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# Abstract

A cytokine storm(CS) is an out-of-control inflammatory response closely associated with the progression of diseases, such as multiple organ failure(MOF), severe sepsis, and severe or critical COVID-19. However, there is currently a lack of reliable diagnostic markers to distinguish CS from normal inflammatory responses. Tumor necrosis factor-α (TNF-α) includes transmembrane TNF-α (tmTNF-α) and secreted TNF-α (sTNF-α). The MOF mouse model in this study showed that the tmTNF-α expression changes in the neutrophils differed from the serum TNF-α and serum IL-4, IL-6, IL-10, and IL-18 and it was the tmTNF-α, instead of serum TNF-α, IL-4, IL-6, IL-10, and IL-18, that reflected the liver and kidney tissue damage and increased with the aggravation of these injuries. Analysis of the ROC results showed that tmTNF-α effectively distinguished between inflammatory response and CS and efficiently differentiated between surviving and dead mice. It also significantly improved the diagnostic value of the traditional CRP marker for CS. These results indicated that tmTNF-α expressed in the neutrophil could be used to diagnose CS in MOF mice, providing an experimental basis to further develop tmTNF-α for diagnosing CS patients.


# Introduction

A cytokine storm(CS) is an uncontrolled systemic inflammatory response involving elevated circulating cytokine levels and immune cell over activation, which can be triggered by treatment, pathogenic infections, cancer, and autoimmune diseases[1, 2]. Although the factors driving CS may differ, late-stage clinical manifestations converge and often overlap[3].Under normal stress, the inflammatory response mediated by increased cytokines helps control infection, which is beneficial for the body to maintain the stability of the internal environment[4]. However, uncontrolled inflammatory responses can mediate CS that can cause severe damage to the host, including acute respiratory distress syndrome, kidney failure, acute liver injury, multiple organ failure(MOF),and even death[2]. Therefore, accurately identifying inflammatory responses and CS is vital for treating patients with associated diseases with selective anti-inflammatory and anti-inflammatory therapies.

With the continuous outbreak of the Ebola virus, avian influenza, SARS coronavirus, and novel Coronavirus in recent years, the role of CS in patients with sepsis, MOF, and severe pneumonia has become a significant concern[5, 6]. Defining the normal inflammatory response and CS have attracted considerable attention for preventing and treating severe patients. The tumor necrosis factor-α(TNF-α), interleukin-18(IL-18), interleukin-6 (IL-6),interferon-γ(IFN-γ), interleukin-4(IL-4),andinterleukin-10(IL-10)cytokines represent the key mediators driving CS. Therefore, clinical attempts have been made to distinguish CS by detecting cytokine levels[7]. Due to the short half-life of cytokines, the changes in the cytokine content are not completely consistent with the severity of the disease. It is difficult to distinguish a CS from an inflammatory response by detecting cytokine levels in the systemic circulation. Furthermore, it cannot accurately reflect the severity of the disease or confirm the diagnostic threshold of a CS[5]. In recent years, some studies have shown that the content changes in non-specific inflammatory markers, such as C-reactive protein(CRP), are related to the severity of the disease and can be used for the auxiliary

detection of CS[8, 9]. However, the sensitivity and specificity of these markers for diagnosing CS still do not meet the needs of clinical diagnosis and treatment.

TNF-α is divided into transmembrane TNF-α(tmTNF-α) and soluble TNF-α(sTNF-α). tmTNF-α is a precursor of sTNF-α, expressed as 26kd on the cell membrane and cleaved to sTNF-α (blood TNF-α) via the TACE enzyme[10]. However, the role of tmTNF-α in disease development differs from sTNF-α. Moreover, tmTNF-α is often difficult to detect or can only be detected at low levels, even upon stimulation, due to being cleaved chiefly by the TNF-α converting enzyme[11]. Several studies have shown abnormally increased tmTNF-α expression intheneutrophils during acute inflammation[12],in non-small cell lung cancer[13], and primary breast cancer[14]. Therefore, the role of tmTNF-α in diseases has attracted increasing attention. This study aims to elucidate the diagnostic value of tmTNF-α in CS involving MOF.

# Materials And Methods

### Experimental animals

The animal study was approved by the Animal Care and Use Committee of Guizhou University of traditional Chinese Medicine. Six- to seven-week-old male C57BL/6 mice were purchased from Sibefu Biotechnology (Bei Jin, China). The animals were housed in pathogen-free conditions and allowed free access to water and food.

### Animal model preparation

Liver failure was induced via intraperitoneal injection with 10μg/kg LPS (Sigma-Aldrich; *Escherichia coli* 0111:B) and 500 mg/kg D-gal (Sigma-Aldrich). Liver tissues and blood samples were then collected before (0 h) and after (2 h, 4 h, and 6h) injection.

### Flow cytometry

Here, 50μLof blood was incubated at room temperature for 40 min with 5μLof a PE-conjugated anti-mouse TNF-α antibody, after which 300μL stromatolyser-4DL FFD-201A was added for 5 min, followed by 500μLPBS.The mixture was centrifuged at 2500g for 5min, after which the supernatant was discarded, and 300μLPBS was added for resuspension. The stained cells were analyzed using an LSR II flow cytometer (Becton Dickinson, San Jose, CA, USA).

### Enzyme-linked immunosorbent assay(ELISA)

The TNF-α, IL-18, IL-6, IL-10,IL-4, and CRP levels were measured using commercial ELISA kits (HePeng Biological) according to the protocols of the manufacturer.

### Histology

The liver and kidney tissues were fixed with 10% formalin for 24 h and then embedded in paraffin wax. For morphological evaluation, the tissue sections (4μm) were routinely stained with hematoxylin-eosin

(H&E).

### The detection of liver and renal function

The ALT and AST levels of the creatinine(Cr) and cystatin C(CysC)were measured using a Mindray ExC8100 system in the clinical laboratory of the Second Affiliated Hospital, Guizhou University of traditional Chinese Medicine.

### Statistical analysis

The data were shown as the median with standard deviation. The differences between the two groups were assessed via Mann–Whitney tests(unpaired tests). A value of $P<0.05$ was considered statistically significant. An AUC was used to sensitively and specifically determine the predictive values of the tested biomarkers to discriminate between the mice with liver failure (6h after injection) and the HC group and between themice displaying an inflammatory reaction and CS. All statistical analyses were performed using GraphPad Prism V9 software.

## Results

### The tmTNF-α expression level in the neutrophils was significantly increased in mice with MOF

The liver and kidney tissues of the animals in the MOF mouse model were severely damaged 6h after LPS/D-gal injection, showing distinctly abnormal functionality, which was consistent with the clinical characteristics of MOF patients(**Supplementary Figs. 1a**, **1b**, **2a**, and **2b**). The tmTNF-α expression levels in the peripheral blood neutrophils of the mice treated 6h after LPS/D-gal injection was significantly higher than in the control mice**(P<0.0001; Figs.1a and 1b)**.

### The tmTNF-α expression changes in the neutrophils differed from the serum cytokines in the MOF mouse model

This study showed that the dynamic tmTNF-α expression changes in the MOF mouse model differed from the serum cytokines. At 2h and 4h after LPS/D-gal injection, the tmTNF-α expression in the neutrophils did not display a substantial increase but was significantly higher after6h, with a value of $P<0.0001$ compared with the control group and $P<0.001$ compared with the 4hsample group(**Fig.2a**). Furthermore, the tmTNF-α expression changes were consistent with those denoting liver tissue damage and liver function and increased with aggravated liver tissue damage. They also corresponded with kidney tissue damage and renal function changes. The changes in the serum TNF-α levels were not associated with liver and kidney tissue injury and peaked 2 h after injection while decreasing to normal levels 6 h after treatment**(Fig. 2b)**. The other serum cytokines, IL-18, INF- γ, IL-4, and IL-6, were significantly higher 2h after injection, reaching the highest levels at 4h or 6h after injection. However, no significant changes were evident in the other cytokines, except for IL-18 6h after injection compared with 4h **(Figs.2c, 2d, 2e, and 2f)**.These content changes were not consistent with those in liver and kidney

tissue injury. These results suggest that tmTNF-α displays a higher value than serum cytokines for diagnosing CS.

### The tmTNF-α expression level in the dead mice differed from the surviving mice

The mice were divided into the death and survival groups according to the survival rate at 6 h after LPS/D-gal injection. The relationship between tmTNF-α and the disease severity caused by CS was further clarified by examining the tmTNF-α expression differences between the surviving and dead mice, while the TNF-α, IL-18, INF- γ, IL-4, and IL-6 differences between the two groups were also compared. The results showed that the tmTNF-α expression level in the dead mice was significantly higher than in the surviving mice (**P<0.0001; Fig. 3a**). Except forIL-18, TNF-α, INF- γ, IL-4, and IL-6, no significant differences were evident between the serum cytokine levels of the two groups**(Figs.2b, 2c, 2d, 2e, and 2f)**, suggesting that the tmTNF-α expression level could distinguish the disease severity caused by CS.

### The evaluation of the diagnostic value of tmTNF-α and IL-18 for CS in MOF mice

The pathological MOF process involves normal inflammatory responses and CS. In the MOF mouse model, the serum cytokine concentrations were high 4hafter LPS/D-gal injection, but the liver and kidney tissues were less damaged, which was consistent with the characteristics of normal inflammatory response and was considered the inflammatory reaction stage. After 6h, the serum cytokine concentrations were high, and liver and kidney tissues were severely damaged, while the liver and kidney function were noticeably abnormal, which was consistent with the characteristics of CS, and was regarded as the CS stage. The ROC curve results showed that tmTNF-α differentiated between inflammatory responses and CS with an AUC value of 0.96(95% CI, 0.92-1.00),a sensitivity of 89.29%, and a specificity of 89.29%**(Fig. 4a)**, while the IL-18 AUC value was 0.63 (95% CI, 0.48-0.77) with 40.00% sensitivity and 85.19% specificity**(Fig. 4b)**.The AUC value of tmTNF-α significantly exceeded that of IL-18in distinguishing between inflammatory responses and CS. Compared with the inflammatory reaction stage, the serum cytokines in the CS stage were statistically significant, except for IL-18, while TNF-α, INF-γ, IL-4, and IL-6 displayed no statistical importance. It is suggested that tmTNF-α is superior to other serum cytokines in distinguishing between inflammatory responses and CS.

To further demonstrate the diagnostic value of tmTNF-α in CS, the AUC values of tmTNF-α and IL-18 were calculated to distinguish the living and dead mice. The results showed that tmTNF-α differentiated the living and dead mice with an AUC value of 0.93 (95% CI, 0.88-0.99), 90.14% sensitivity,and 80.00% specificity**(Fig. 5a)**, while IL-18 exhibited an AUC value of 0.62 (95% CI, 0.47-0.76),a sensitivity of 80.65%, and a specificity of 46.43%**(Fig. 5b)**. These results suggest that tmTNF-α displays potential diagnostic value for the disease severity caused by CS.

### Combining tmTNF-α and the traditional marker, CRP, can improve their value for CS diagnosis

In clinical diagnosis, CRP is a standard indicator of the disease severity caused by CS. This study assessed the value of tmTNF-α combined with CRP for diagnosing CS. The results indicated that CRP

differentiated between CS and inflammatory responses with an AUC value of 0.84 (95%CI,0.73-0.94),64.29% sensitivity, and 92.86% specificity**(Fig. 6a)**, which was lower than tmTNF-α alone **(Fig. 6b)**. The tmTNF-α and CRP combination substantially improved the diagnostic efficiency (AUC=0.98, 95% CI:0.95-1.00),while the sensitivity and specificity were 92.86% and 92.86%, respectively (**Fig. 6c**). Similarly, when comparing the living and dead mice, the CRP displayed an AUC of 0.82 (95% CI, 0.70-0.94), 67.86% sensitivity,and 92.86% specificity (**Fig. 6d**), which was lower than tmTNF-α alone **(Fig. 6e)**. Furthermore, the diagnostic ability of the tmTNF-α and CRP combination was significantly improved, with an AUC value of0.98(95%CI,0.94-1.00), 89.29% sensitivity, and 89.29% specificity **(Fig. 6f)**.

# Discussion

No single definition of CS is commonly accepted, while there is disagreement about how these disorders differ from an appropriate inflammatory response[3]. However, studies have shown that CS, in addition to high serum cytokine levels, are often accompanied by organ dysfunction and histopathological damage, which are closely related to the occurrence of acute liver failure, MOF, and sepsis[15–17].In recent years, research has indicated that severe Ebola, avian influenza, SARS coronavirus, and novel coronavirus infections are also associated with the occurrence of CS[18, 19]. However, a reliable method for diagnosing CS is still lacking.

Although tmTNF-α is a precursor of sTNF-α, the disease changes differ from those of sTNF-α (blood TNF-α)[10].In the MOF mouse model, the blood TNF-α at 2 h after LPS/D-gal injection was significantly higher than the normal control but decreased to normal levels at 6h. However, the tmTNF-α expression differed from the blood TNF-α, which was relatively low at 4h after injection but was substantially higher at 6h, increasing with the deterioration of the damaged liver and kidney tissues. Common clinical markers for CS diagnosis include IL-6, IFN-γ, IL-4, and IL-18[20–24]. Here, changes in the blood levels of these cytokines were different from those in tmTNF-α, which, despite their high levels, were not consistent with the severity of liver and kidney tissue injury. The expression levels of these cytokines did not reach a maximum in the most severe liver tissue damage cases. Except for IL-18, no significant differences were evident in serum cytokines between 6h and 4h after LPS/D-gal injection, nor between the living and dead mice. Significant differences were evident between the tmTNF-α expression levels at 6h and 4h after injection, as well as between the survival and death rates of the mice, suggesting that tmTNF-α displayed potential for diagnosing CS.

ROC curve analysis is a commonly used statistical method in clinical diagnostic tests[25, 26]. The diagnostic ability of test indexes can be directly judged according to the AUC value. To clarify the diagnostic ability of tmTNF-α, this study calculated the AUC value of tmTNF-α for CS, while using IL-18 as a control. The liver and renal tissue damage were mild 4h after LPS/D-gal injection, with slightly abnormal liver and renal function, which was used as the normal inflammatory reaction stage. However, 6h after this treatment, the liver tissue and renal tissue damage were severe, while the functionality of these organs was highly abnormal, representing the CS stage. ROC curve analysis showed that the tmTNF-α AUC value was significantly higher than IL-18, suggesting that tmTNF-α could effectively

distinguish between the inflammatory response and CS. Furthermore, this study evaluated the AUC value of tmTNF-α in distinguishing between the dead and surviving mice to assess its role in mitigating the disease severity caused by CS, indicating that the AUC value was significantly higher than IL-18. This suggests that the tmTNF-α expression level can effectively alleviate the disease severity resulting from CS.

Although CRP is a non-specific marker for diagnosing CS[27–29], its specificity and sensitivity levels do not meet clinical needs[30]. Here, we found that tmTNF-α in combination with CRP significantly increased, compared to the value of CRP alone in differentiating inflammatory responses from CS, as well as in distinguishing alive and dead mice. ROC curve analysis showed that CRP differentiated CS from inflammatory responses with an AUC value of 0.84 (95%CI, 0.73–0.94), while the tmTNF-α and CRP combination significantly increased the diagnostic value with an AUC value of 0.98 (95% CI, 0.95-1.00). Similarly, the AUC value of CRP was only 0.82 (95% CI, 0.70–0.94)in differentiating between the surviving and dead mice, while the combined CRP displayed a significantly higher AUC value of 0.98(95%CI,0.94-1.00). These results imply that tmTNF-α can improve the diagnostic efficiency of CRP.

This study indicated that the tmTNF-α expression in the peripheral blood neutrophils can be used to diagnose CS and assess their severity. However, at present, it is only limited to animal experiments. It is not clear whether the expression of neutrophils in the peripheral blood of patients with MOF involving CS is increased and whether the expression level is related to disease severity. Moreover, whether the tmTNF-α expression in the peripheral blood neutrophils of patients with different degrees of CS display similar results requires further investigation.

# Declarations

### AUTHOR CONTRIBUTION

Peng Yang and Ruping Zhang planned and executed the experiments; Yimin Zeng, Xin Peng, Fang Yang, Yongsheng Hu and Xuhong Tan participated in the study design, collated data, analyzed data; Peng Yang and Ruping Zhang drafted and wrote the manuscript. All authors read and approved the final submission.

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### DATA AVAILABILITY

Not applicable.

### Ethics Approval

This study was authorized by the Ethics Committee of Guizhou University of Traditional Chinese Medicine (20210074).

### Consent to Participate

Not applicable.

### Consent for publication

Not applicable.

### Competing Interests

The authors declare no competing interests.

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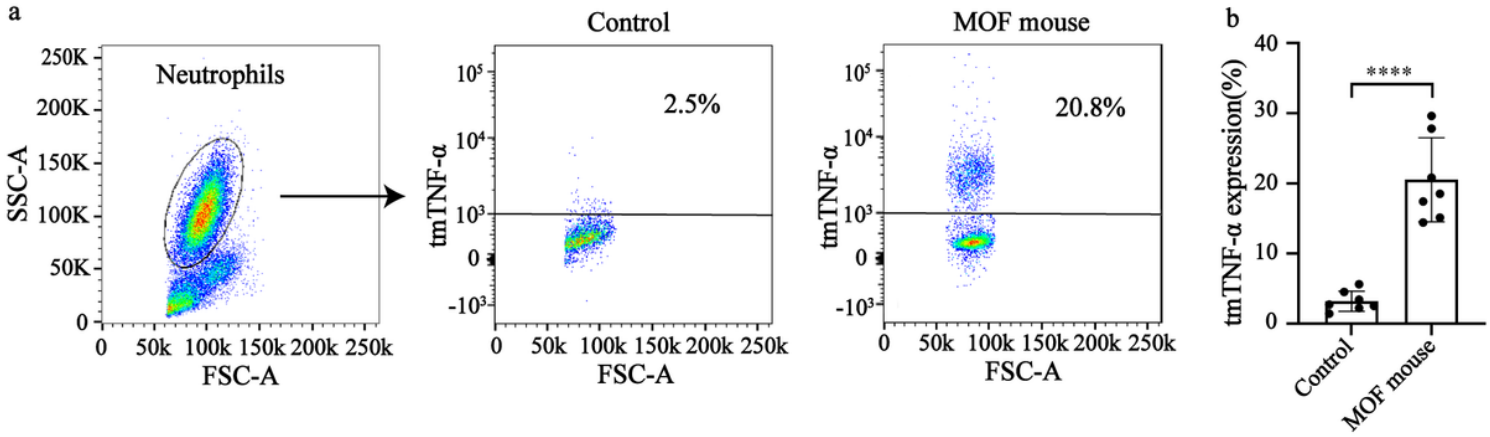
# Figures




## Figure 1

The tmTNF-α expression level in the blood neutrophils of the control group and MOF mouse model.**(a)**A representative FCM image of blood neutrophils. **(b)**Quantitative analysis ofthe blood neutrophils.****indicates the comparison between the two groups, P<0.0001.

## Figure 2

The dynamic tmTNF-α expression changes in the neutrophils and serum cytokines in the mice with MOF. **(a)** The tmTNF-α expression changes in the peripheral blood neutrophils. The changes in**(b)**TNF- α, **(c)** IL-18, **(d)** INF- γ, **(e)** IL-4,and**(f)** IL-6 content at different time points after LPS/D-gal injection. The treatment group was compared with the control group at different times after LPS/D-gal injection.* indicates P<0.05, *** indicates P<0.001, and **** indicates P<0.0001. Compared with mice treated 4h and 6h after LPS/D-gal injection, ## indicates P<0.01, and ####indicates P<0.0001.

## Figure 3

of the tmTNF-α expression levels in the neutrophils and serum cytokines in the dead and surviving mice. **(a)**The tmTNF-α expression level in the peripheral blood neutrophils. The **(b)**TNF-α,**(c)** IL-18, **(d)** INF-γ,**(e)** IL-4, and **(f)**IL-6contentinthe dead and surviving mice.

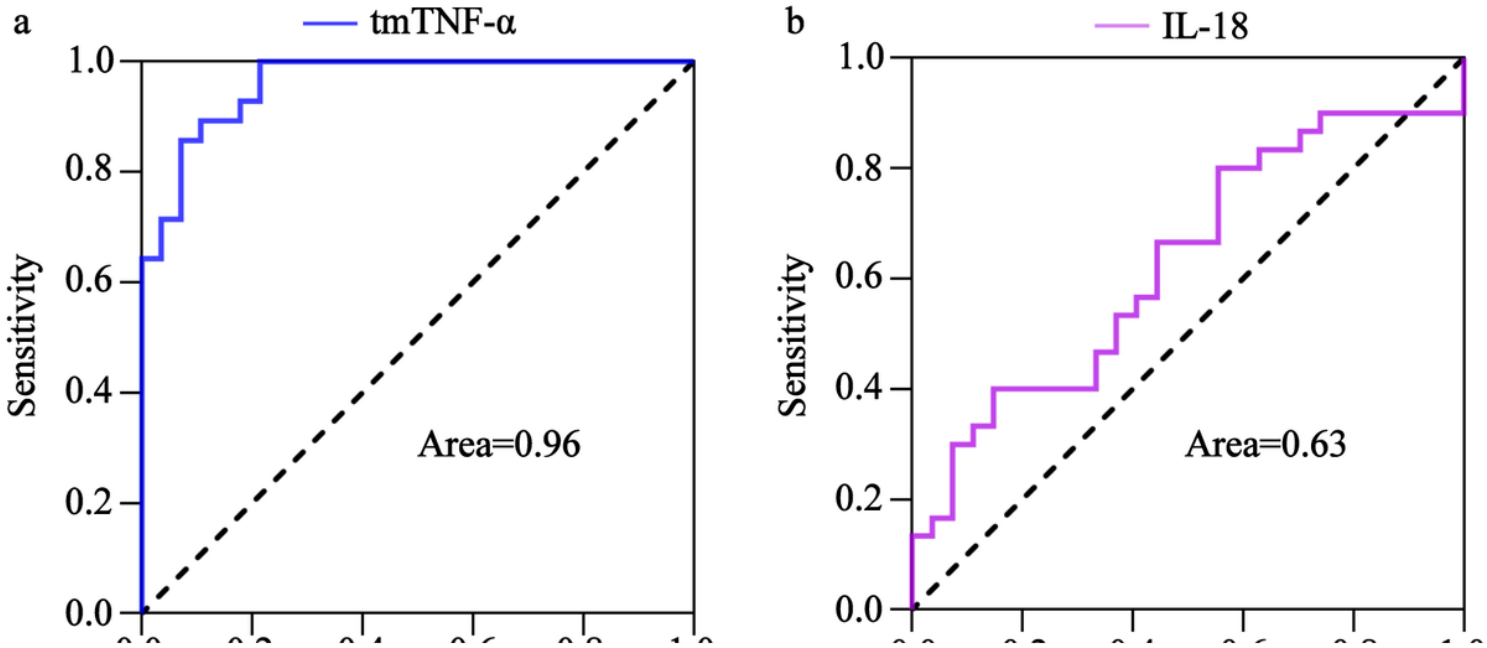



**Figure 4**

The tmTNF-α and IL-18 ROC curve for distinguishing between the CS and inflammatory response. The ROC curves of **(a)**tmTNF-α and **(b)**IL-18 during the differentiation between inflammatory response and CS.

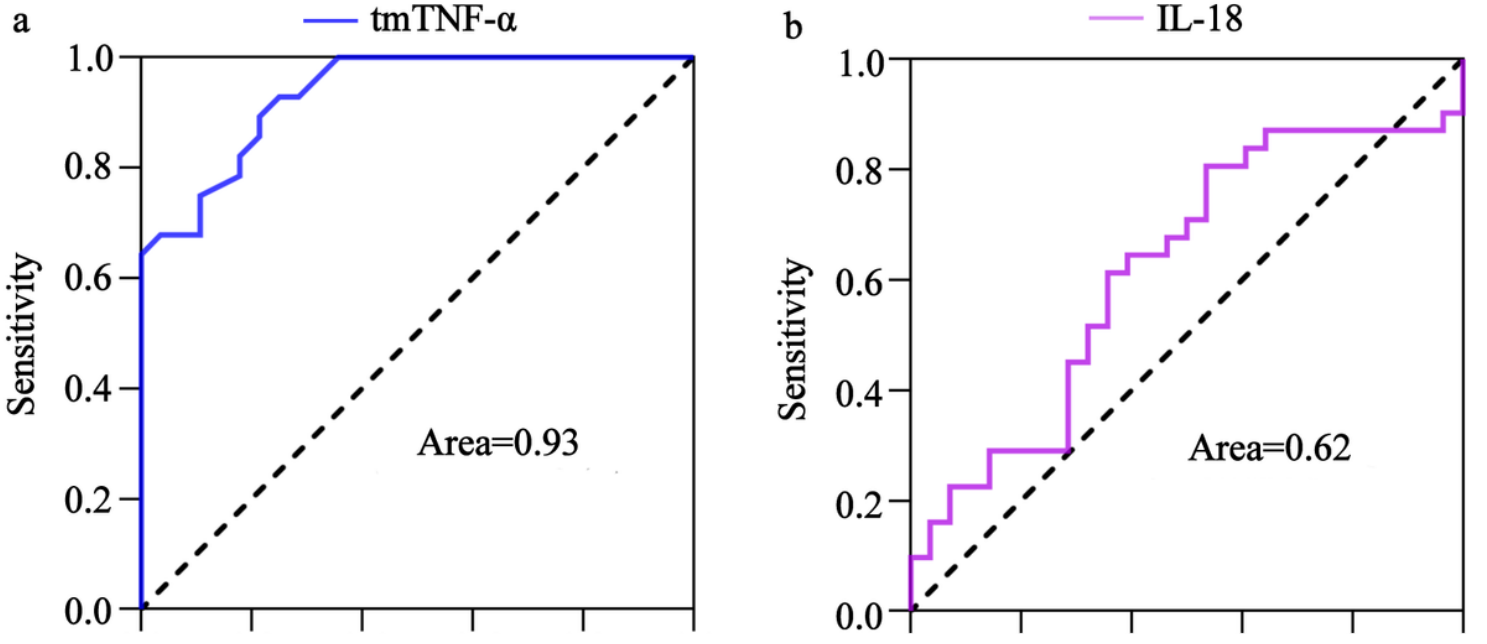



**Figure 5**

The tmTNF-α and IL-18 ROC curves for distinguishing between CS and inflammatory response. The ROC curve of **(a)**tmTNF-α and **(b)**IL-18 when differentiating between the dead and surviving mice.

**Figure 6**

The ROC curve of the tmTNF-α and CRP combination for diagnosing CS. The ROC curves of the **(a)**tmTNF-α, **(b)**CRP, and **(c)**tmTNF-α and CRP combination for distinguishing between the inflammatory response and CS. The ROC curves of the **(d)**tmTNF-α, **(e)**CRP, and **(f)** tmTNF-α and CRP combination for distinguishing between the dead and surviving mice.

# Supplementary Files

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