High expression levels of trigger receptor expressed on myeloid cells-1 on neutrophils associated with increased severity of acute pancreatitis in mice

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Summary

Triggering receptor expressed on myeloid cells (TREM)-1 expression on neutrophils is associated with inflammation and infection. However, the dynamic changes of the TREM-1 expression on neutrophils have not been clarified in inflammatory acute pancreatitis (AP). The aim of this study was to longitudinally investigate the TREM-1 expression on peripheral blood and peritoneal neutrophils and its relationship with the levels of plasma cytokines and disease severity in a mouse model of AP following injection with varying doses of L-arginine to induce mild or severe AP. The results indicated that induction of MAP or SAP was associated with moderate and severe pancreatic tissue damage and varying levels of serum and peritoneal fluid amylase as well as survival rates in mice. In comparison with that in the healthy controls, significantly increased percentages of peripheral blood and peritoneal fluid CD14-TREM-1+ neutrophils and higher levels of TREM-1 mRNA transcripts in peripheral blood nuclear cells were detected in the MAP and SAP mice, particularly in the SAP mice. Higher levels of plasma TNF-α and GM-CSF, but lower levels of plasma IL-10, were detected in the MAP and SAP mice at varying time points post induction. The percentages of peripheral blood CD14-TREM-1+ neutrophils were correlated positively with the levels of TNF-α, GM-CSF, and amylase as well as the pathogenic scores, but negatively with the levels of IL-10 in the AP mice. Therefore, TREM-1+ neutrophils may participate in the pathogenesis of AP and serve as a biomarker for evaluating the severity of AP.

Keywords: Acute pancreatitis; TREM-1; animal models; inflammatory; cytokine
Introduction

Acute pancreatitis (AP) is a sudden inflammation of the pancreas and also affects other organs. The severity of AP symptoms varies from mild abdominal discomfort to a severe, life-threatening illness; \(^1\) approximately 20% of patients develop severe AP (SAP) with a high rate of mortality \(^2, 3\). Mild acute pancreatitis (MAP) is usually self-limited and can be resolved within a few days with conservative treatment. However, nearly half SAP patients die in the first week due to multiple organ dysfunction syndrome (MODS). During the pathogenic process of SAP, aberrant activation of inflammatory cells and excessive production of inflammatory mediators such as tumor necrosis factor (TNF)-\(\alpha\), interleukin (IL)-6, and granulocyte-macrophage colony stimulating factor (GM-CSF) can cause pancreatic necrosis and systemic inflammatory response syndrome (SIRS) and MODS, which are the leading causes of morbidity and mortality.\(^1, 4, 5\) Other affected individuals may die within weeks or months due to severe complications and septicemia.\(^6\) While early intervention of SAP patients may save their lives, there are few reliable biomarkers precisely to diagnose SAP at early stage.\(^7, 8\)

Currently, early diagnosis of SAP depends on clinical symptoms, imaging, and laboratory examination of patients.\(^9\) However, the values of some clinical measures may not reflect the severity and progression of SAP; for example, elevated levels of serum pancreatic enzymes are not well correlated with these parameters. Moreover, serum amylase level is commonly transiently elevated and declines within a short period after onset.\(^10-12\) Therefore it is very important to reveal the pathogenesis of SAP and discover new biomarkers for evaluating its severity.

Triggering receptors expressed on myeloid cells-1 (TREM-1) is selectively expressed on neutrophils and a subset of CD14\(^+\) monocytes.\(^13, 14\) TREM-1 expression can be induced by bacterial infection or endotoxin, and is proposed as a diagnostic marker of
Clinical studies have shown that the numbers of TREM-1\(^+\) cells can be used to evaluate the severity of sepsis or SIRS.\(^{17}\) Indeed, dynamic changes in levels of serum TREM-1 are associated with the prognosis of patients with sepsis.\(^{16}\) However, another study showed that measurement of soluble TREM-1 does not increase the diagnostic accuracy in patients with SIRS.\(^{18}\) TREM-1 is involved in monocyte activation and inflammatory response via inducing pro-inflammatory cytokines such as TNF-\(\alpha\), IL-6, and GM-CSF but inhibiting IL-10 production. During the pathogenesis of SAP, these cytokines can promote production of other pro-inflammatory cytokines including IL-1, and IL-8, enhancing inflammatory response and tissue damage.\(^{19}\) In contrast, IL-10 inhibits inflammatory responses and cerulean-induced pancreatic injury.\(^{20}\) However, dynamic changes in the numbers of TREM-1\(^+\) neutrophils and levels of TNF-\(\alpha\) and IL-10 during the pathogenic process of SAP are unclear.

There are several experimental animal models of AP available for research on the pathogenesis, potential biomarkers, and therapeutic strategies of AP. In particular, the non-invasive model of AP induced by high-dose L-arginine has been demonstrated valuable.\(^{21}\) Furthermore, the severity of AP induced by L-arginine is dose- and time-dependent, which is very useful for the investigation of early and late phases of the process of AP.\(^{22-24}\) In addition, the course, severity, and histological changes of AP in this model are similar to those in humans.

In the present study we employed mouse models of MAP and SAP induced by varying doses of L-arginine to determine dynamic changes in TREM-1 expression longitudinally and to evaluate potential associations between levels of TREM-1 expression and disease severity in this model. The aim of our study was to determine whether TREM-1 could be used as a novel biomarker to evaluate the severity of AP at
early stage.

Materials and methods

Animals

Female BABL/c mice at 8 weeks of age and 20-24 g were purchased from the Experimental Animal Center of Anhui Medical University. Animals were maintained in a specific pathogen-free facility with constant temperature of 25°C, light-dark cycle 12/12 h, and free access to water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Anhui Medical University.

Animal model

A mouse model of AP was induced by intraperitoneal injection of high-dose L-arginine as described previously.21 Briefly, mice were randomly injected intraperitoneally (i.p.) with 2.5 or 3.0 g/kg L-arginine (20% in saline; Sigma, St. Louis, MO, USA) to induce MAP or SAP, respectively (n=40/group). Mice in MAP or SAP group were re-injected with the same dose of L-arginine at 1 h after the first injection. Mice were sacrificed at 6, 12, 24, 48, or 72 h after the second injection (n=8/group/time-point). A control group of mice was injected i.p. with saline alone (n=8). Before sacrifice, the mice were anesthetized and injected i.p. with saline 1 mL for collecting peritoneal lavaging fluid. The collected peritoneal lavaging fluid samples were centrifuged at 1200g for 10 min. Their supernatants were collected for biochemistry analyses and the pelleted cells were used for flow cytometry analysis. Blood samples were obtained for preparing plasma samples and pancreata collected for histological examination.
**Determination of amylase**

Concentrations of plasma amylase in individual mice were determined by Olympus AU2700 Chemistry Analyzers.

**Flow cytometry analysis**

Expression of TREM-1 on peripheral blood leukocytes and peritoneal cells was determined by flow cytometry. Briefly, peripheral blood and peritoneal fluid cells (5 × 10^5/tube) were treated in duplicate with 10% rat sera in staining buffer at 4°C for 1 h and after washing, red blood cells were lysed by erythrocyte lysing kit (eBioscience, San Diego, CA, USA) and the remaining leukocytes stained with phycoerythrin (PE)-conjugated anti-TREM-1 (R&D, Minneapolis, MN, USA), FITC-conjugated anti-CD14 (eBioscience), and APC-conjugated anti-MHC class II (eBioscience) antibodies on ice for 30 min. After washing, the cells were fixed with 2% paraformaldehyde (PFA) and characterized by flow cytometry analysis using a FACS Calibur flow cytometry system (BD Immunocytometry Systems, Franklin Lakes, NJ, USA). The cells were gated on polynuclear cells. The frequency of CD14^+ TREM-1^+ neutrophils and intensity of TREM-1 expression on neutrophils were characterized by flow cytometry. At least 20,000 events were analyzed for each sample.

**Semi-quantitative RT-PCR**

Total RNA was extracted from individual peripheral blood leukocyte samples using Trizol reagent (Invitrogen, Paisley, UK), according to the manufacturer's instructions. The RNA samples were reversely transcribed into cDNA using random hexamer primer and a reverse transcription reagent kit according to the manufacturer's instructions (Fermentas, Glen Burnie, MD, USA). The levels of target mRNA transcripts were determined by semi-quantitative PCR using the cDNA as the
template and specific primers. The sequences of primers were: sense
5'-GGGGACTGCTGTGCGTGTT-3' and antisense
5'-GTGGGCTTGGGTAGGGAT-3' for TREM-1; sense
5'-CACGATGGAGGGCCGGACTCATC-3' and antisense
5'-TAAAGAGCCAACACAGT-3' for β-actin. The PCR reactive system (25 μL/each) contained 2.5 μL MgCl₂ (25 mM), 2.5 μL 10×Taq buffer, 1 μL dNTP mixture (10 mM), 1 μL primers mixture, 0.5 μL Taq DNA polymerase, 5 μL cDNA, and 12.5 μL nuclease-free water. The PCR reactions were performed in duplicate at 95°C for 3 min and subjected to 30 cycles of 95°C for 45 s, 55.7°C for 45 s, and 72 °C for 1 min. The PCR products were resolved on agarose gel electrophoresis and analyzed by densitometric scanning.

**Enzyme-linked immunosorbent assay (ELISA)**

Concentrations of plasma TNF-α, GM-CSF, and IL-10 were determined by ELISA using specific kits according to the manufacturer's instructions (Shanghai Senxiong Biotech, Shanghai, China). The limit of detection for TNF-α, GM-CSF, and IL-10 was 7.8, 1.0, and 8.3 pg/mL, respectively.

**Pancreatic histopathology**

Individual pancreata were fixed in 4% paraformaldehyde overnight and permeabilized with dimethylbenzene then embedded in paraffin. The pancreatic tissue sections (5 μm) were stained with hematoxylin (H) and eosin (E). The stained sections were examined under a light microscope and images captured. The severity of edema, inflammation, vacuolization, and necrosis in the pancreatic tissues of individual mice was evaluated and each item graded a score 0-4 (normal to severe) as described previously in a blinded manner. Individual mice with SAP were identified if they had a pathological
score ≥8 whereas those with MAP were determined if they had a pathological score <8 at 48 h after the second injection with L-arginine.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences among groups were analyzed by one way analysis of variance (ANOVA) and Student’s t-test. Relations between variants were assessed by linear correlation analysis using SPSS software (for Windows, version 13). A p-value <0.05 was considered statistically significant.

Results

Induction of MAP and SAP in mice

To determine pathogenesis of MAP and SAP, BALB/c mice were injected with different doses of L-arginine and the development of clinical symptoms was monitored longitudinally. Following injection, the mice became sluggish and exhibited polydipsia; their skin became pale and clammy in the MAP and SAP groups. Furthermore, mice in SAP group had abdominal distension, tachypnea, and peripheral cyanosis. During the 72-h observation period, 10 mice in SAP group and five in MAP group died. Survival rate in the control, MAP, and SAP groups was 100%, 87.5%, and 75%, respectively (Fig. 1A); the difference in this parameter between the SAP and MAP was not statistically significant.

At 6 h after induction in the MAP group, pancreatic tissues displayed edema but no peritoneal exudates. In contrast, in pancreatic tissues of the SAP group massive abdominal hemorrhagic exudates, obvious pancreatic edema, and intestinal hyperemia
were found. Histological examination indicated typical changes of acute inflammation with intensive edema and inflammatory infiltrates in pancreatic tissues in both the MAP and SAP groups. Intensive necrosis, evident leukocyte infiltration, focal hemorrhage, and severe acinar cell damage were observed in pancreatic tissues of the SAP group, but not obviously in the MAP group (Fig. 1C). Pathological scores in the MAP and SAP groups increased over time and were significantly greater in the SAP than the MAP group (Fig. 1B). Serum amylase was significantly increased in the SAP and MAP groups versus control at 6 h post-injection and higher levels of serum amylase were detected at 48 and 72 h post-induction, particularly in the SAP group (Fig. 1D). A similar pattern of amylase in peritoneal lavage fluid was observed (Fig. 1E). The clinical symptoms, pathogenic characteristics, and elevated levels of amylase demonstrated the development of AP in mice.

Higher levels of TREM-1 expression on peripheral blood and peritoneal neutrophils in AP mice

To determine the potential role of TREM-1⁺ neutrophils in the pathogenesis of AP, the frequency of peripheral blood and peritoneal lavage fluid CD14⁻ TREM-1⁺ neutrophils and the intensity of TREM-1 expression on neutrophils in the different groups of mice were characterized longitudinally by flow cytometry (Fig. S1, Fig. 2). Quantitative analyses indicated that the percent peripheral blood CD14⁻ TREM-1⁺ neutrophils was significantly higher in the MAP and SAP groups versus control at 6 h post-induction (p<0.01) and gradually declined in the MAP group thereafter. In contrast, in SAP group the frequency of peripheral blood CD14⁻ TREM-1⁺ neutrophils remained at high levels until 48 h post-induction and declined at later time-points. The percent peripheral blood CD14⁻ TREM-1⁺ neutrophils in the SAP group was significantly higher than in the MAP group at 24, 48, and 72 hrs post-induction (p<0.05 or <0.01). Similarly, the percent peritoneal lavage fluid CD14⁻ TREM-1⁺ neutrophils in SAP group was significantly higher than in MAP group at 6, 48, and 72
h post-induction. In addition, the intensity of TREM-1 expression on neutrophils was significantly higher in the SAP versus MAP group.

We further characterized TREM-1 expression on peripheral blood leukocytes by semi-quantitative RT-PCR. The relative levels of TREM-1 mRNA transcripts in peripheral blood leukocytes from the MAP group were significantly higher than in control and significantly lower than SAP animals at 24 h post-induction (p<0.05; Fig. 3A). Notably, the percent CD14-TREM-1+ neutrophils was positively correlated with levels of serum amylase (r=0.422; p=0.000) and pancreatic pathological scores (r=0.623; p=0.000) in AP mice (Fig. 3B and 3C). Hence higher levels of TREM-1 expression on neutrophils are associated with disease severity in AP mice.

**Imbalance of plasma TNF-α, GM-CSF, and IL-10 associated with higher percent peripheral blood CD14+ TREM-1+ neutrophils in AP mice**

Pro-inflammatory cytokines such as TNF-α and GM-CSF participate in the pathogenesis of AP whereas inhibitory IL-10 negatively regulates progression of AP. To determine the relations between TREM-1 expression and plasma cytokines the concentrations of plasma TNF-α, GM-CSF, and IL-10 in the different groups of mice were determined longitudinally by ELISA (Fig. 4A-C). Significantly increased levels of plasma TNF-α and GM-CSF were detected in MAP and SAP groups versus control and these concentrations were significantly higher in the SAP versus MAP group at most time-points. In contrast, concentrations of plasma IL-10 in the MAP and SAP groups were significantly lower than control at most time-points and significantly lower in SAP versus MAP group at 72 h post-induction. Apparently, higher levels of pro-inflammatory cytokines and lower levels of inhibitory IL-10 are associated with the development of AP in mice.
The percent peripheral blood CD14− TREM-1+ neutrophils was positively correlated with concentrations of plasma TNF-α (r=0.504; p=0.000) and GM-CSF (r=0.485; p=0.000) but negatively with levels of plasma IL-10 (r=-0.722; p=0.000) in AP mice (Fig. 4D-F).

Discussion

TREM-1, a member of the Ig superfamily, is expressed on myeloid cells and mediates activation of neutrophils and monocytes. Previous studies have shown that dynamic changes in TREM-1 expression are associated with the severity and prognosis of sepsis. Inhibition of the TREM-1 pathway can reduce inflammatory responses without affecting pathogen clearance, and prolong the survival of animals following infection. More importantly, levels of TREM-1 mRNA expression were negatively correlated with the severity of sepsis in human patients. In this study we employed animal models of AP induced by injection with different doses of L-arginine to determine levels of TREM-1 expression on peripheral blood and peritoneal lavage fluid neutrophils longitudinally. We found that following induction of AP, levels of TREM-1 expression on neutrophils rapidly increased in MAP and SAP groups whereas the abundance of TREM-1+ neutrophils and relative levels of TREM-1 expression in SAP group were significantly higher versus MAP group animals. Our data are in agreement with a previous observation that expression of TREM-1 on myeloid cells is upregulated in patients with AP. Hence levels of TREM-1 expression may be associated positively with the severity of AP. In parallel, the percent TREM-1+ neutrophils was positively associated with pathological scores, levels of serum amylase, and pro-inflammatory cytokines but negatively with levels of anti-inflammatory IL-10 in mice. Conceivably, higher percent TREM-1+ neutrophils and higher levels of TREM-1 expression on neutrophils may be valuable biomarkers for evaluating the severity of AP.
AP is an inflammatory process in the pancreas that damages acinar cells, leading to activation of intra-pancreatic digestive enzymes, peripancreatic tissue damage, and large quantities of cytokine production associated with the development of SIRS.\(^{34}\)

On the other hand, the inflammatory process of AP is negatively regulated by anti-inflammatory cytokines such as IL-10.\(^{20, 35}\) In this study we detected significantly higher levels of serum TNF-α and GM-CSF and lower levels of IL-10 in MAP and SAP groups versus control and the changes in plasma levels of these cytokines were associated with the severity of AP. Indeed, higher levels of pro-inflammatory cytokines have been noted in individuals with AP.\(^{20, 34, 36}\)

Furthermore, previous studies have shown that exogenous IL-10 or administration of an IL-10 inducer such as insulin-like growth factor (IGF)-1 attenuates pancreatic tissue injury in animal models of AP induced by cerulein.\(^{20, 37}\) Our data support the notion that concentrations of pro-inflammatory cytokines such as TNF-α may be used as an early diagnostic criterion for AP.\(^{36, 38}\) Our findings suggest that an imbalance of pro- and anti-inflammatory cytokines may be associated with the development and progression of AP.

TREM-1 acts as an amplifier of the inflammatory response and cooperates in its initiation.\(^{39}\) TREM-1 is highly expressed in AP patients at early stages of the disease.\(^{40}\) We found significantly increased numbers of TREM-1\(^{+}\) neutrophils and higher levels of TREM-1 expression in MAP and SAP groups and the levels of TREM-1 expression were associated with the severity of AP. Furthermore, the TREM-1\(^{+}\) neutrophil counts were positively correlated with levels of plasma TNF-α and GM-CSF and negatively with IL-10 in AP mice. Our data are in agreement with the notion that activation of TREM-1 promotes production of pro-inflammatory TNF-α, IL-1β, and GM-CSF but inhibits IL-10.\(^{19}\) These findings suggest that TREM-1 expression may be crucial for imbalance of pro-inflammatory and anti-inflammatory responses during the process of AP and TREM-1 may be a
therapeutic target for intervention of AP.

In summary, significantly greater numbers of TREM-1$^+$ neutrophils and higher levels of TREM-1 expression were observed in AP mice, which were associated with imbalance of pro-inflammatory and anti-inflammatory cytokine responses and correlated positively with severity of AP. Our data suggest that levels of plasma pro-inflammatory and anti-inflammatory cytokines and numbers of TREM-1$^+$ neutrophils may provide valuable data for evaluating the severity of AP and are potential targets for therapeutic intervention of AP. Our findings provide novel insights into the pathogenesis of AP.

**Conflict of interest**

The authors declare no conflicts of interest.

**Acknowledgment**

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Figure legends

Figure 1 Establishment of L-arginine-based mouse models of AP.

BALB/c mice were injected with varying doses of L-arginine to induce MAP and SAP. The levels of serum and peritoneal fluid amylase and pancreatic pathologic changes were examined at the indicated time-points post-induction. Survival was monitored. One group of mice (n=8) injected with saline served as control (NC). Data are representative images and expressed as mean ± SD of each group (n=4-8/group) from two separate experiments. (A) Kaplan-Meier survival curves. (B) Pathological scores of pancreatic injury. (C) Representative pancreatic tissue images. Animals were analyzed at 24 h (MAP) or 6 h (SAP) after L-Arginine injection. Upper panel: magnification ×100. Lower panel: magnification ×400. (D) Levels of serum amylase activity. (E) Levels of peritoneal fluid amylase activity (original data logarithmically transformed). MAP, minor acute pancreatitis; SAP, severe acute pancreatitis. *p<0.05; **p<0.01 vs. control. #p<0.05; ##p<0.01 vs. MAP.

Figure 2 TREM-1 expression on neutrophils in peripheral blood and peritoneal lavage fluid.

Peripheral blood and peritoneal lavage fluid samples from individual mice and frequency of TREM-1+ neutrophils and intensity of TREM-1 expression were characterized at the indicated time-points post-induction by flow cytometry. Data are expressed as mean % ± SD of each group of mice (n=4-8/group/time-point) from three separate experiments. (A) Percent circulating TREM-1+ neutrophils. (B) Mean intensity of fluorescence (MIF) of TREM-1 expression on circulating neutrophils. (C) Percent TREM-1+ neutrophils in peritoneal lavage fluid. (D) MIF of TREM-1 expression on neutrophils in peritoneal lavage fluid. *p<0.05; **p<0.01 vs. control.
Figure 3 TREM-1 mRNA expression in peripheral blood cells.

Peripheral blood samples from individual mice and blood leukocytes were obtained. The relative levels of TREM-1 mRNA expression were determined at 24 h after second injection by semi-quantitative RT-PCR. Subsequently, potential relations between relative levels of TREM-1 expression and serum amylase activity or pathological scores of pancreatic inflammation in AP mice were analyzed. Data are representative images and expressed as mean ± SD of each group (n=4-8/group). (A) TREM-1 mRNA expression in peripheral leucocytes. Lanes 1-3, 4-8, and 9-16 are samples from control, MAP, and SAP groups, respectively. *p<0.05 vs. control. (B) Correlation analysis between TREM-1 expression and concentrations of serum amylase. (C) Correlation analysis between TREM-1 expression and pathological scores.

Figure 4 Concentrations of plasma TNF-α, GM-CSF, and IL-10 and their relations with levels of TREM-1 expression.

The concentrations of plasma TNF-α, GM-CSF and IL-10 in individual mice were determined longitudinally by ELISA. Potential relations between levels of cytokines and TREM-1 expression were analyzed. Data are expressed as mean ± SD of each group (n=4-8/group/time-point) from three separate experiments. (A) Levels of plasma TNF-α. (B) Levels of plasma GM-CSF. (C) Levels of plasma IL-10. (D) Correlation between plasma TNF-α and TREM-1 expression. (E) Correlation between plasma GM-CSF and TREM-1 expression. (F) Correlation between plasma IL-10 and TREM-1 expression. *p<0.05; **p<0.01 vs. control. #p<0.05; ##p<0.01 vs. MAP.
Figure 2
Figure 3
Figure 4