ORIGINAL ARTICLE

The Immunomodulatory Effects and Mechanisms of Tim-3 Action in the Early Stage of Mice with Severe Acute Pancreatitis

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ABSTRACT

Background: Tim-3 has been considered as an ideal target for the immunotherapy of inflammation, but it is unclear whether Tim-3 also plays an important role in acute pancreatitis (AP), as well. **Objective:** To identify the immunomodulatory effects and mechanisms of Tim-3 action in the early stages of severe acute pancreatitis in mice. **Methods:** Male BALB/c mice were randomly divided into sham injection group, severe acute pancreatitis group, and anti-Tim-3 treated group. Histopathological scores of the pancreas were calculated, pancreatic myeloperoxidase (MPO) activity was assessed. The concentrations of serum IL-6, IL-10, and TNF-α were evaluated by ELISA kits. Quantitative RT-PCR was performed to detect the transcript amounts of Tim-3, IL-6, IL-10, TNF-α, and TLR4 in peritoneal macrophages. The levels of peritoneal macrophages Tim-3, TLR4, MyD88, and NF-kB p65 were measured by western blot analysis. **Results:** The pathological scores of the anti-Tim-3 treated group (11.5 ± 1.3) significantly increased compared with the sham (1.3 ± 0.5) and SAP groups (6.9 ± 1.0). Furthermore, the downregulation of Tim-3 significantly aggravated mouse pancreatic tissue damage. It was further shown that Tim-3 negatively regulated the production of pro-inflammatory cytokines, IL-6 and TNF-α, as well as anti-inflammatory cytokine IL-10. Of note, the negative regulation of inflammatory cytokines by Tim-3 was mediated by the activation of TLR4/MyD88 NF-kB signaling pathway. **Conclusion:** Our study showed that Tim-3 might play an important role in the development of AP through regulating the inflammatory response.

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INTRODUCTION

The pathogenesis of severe acute pancreatitis (SAP) is a complex, multi-factorial pathophysiological process. The abnormal inflammatory responses play an important role in the initiation and development of SAP. Therefore, the manner of regulating the inflammatory response in SAP by immunomodulation has important clinical significance (1). SAP generally includes the following three stages: systemic inflammatory response syndrome (SIRS), compensatory anti-inflammatory response syndrome (CARS), and mixed anti-inflammatory response syndrome (MARS) (2,3). The changes in the immune system at different SAP stages are associated with different inflammatory factors and cytokines; therefore, it is necessary to perform individualized immunomodulation therapy based on the different stages of acute pancreatitis (AP) (1). In the early stages of SAP, due to the imbalance between anti-inflammatory cytokines and pro-inflammatory cytokines and the excessive production of inflammatory mediators, cytokines are able to trigger a cascade reaction of inflammatory mediators, leading to the rapid development of AP from local lesion to SIRS, resulting in multiple organ failure (MOF) (4,5). Accordingly, it is important to regulate the excessive inflammatory response in the early stages of AP through immunomodulation. Toll-like receptor (TLR) is a pathogen-associated molecular pattern transmembrane receptor with a significant part in pathogen recognition, signal transduction, and immune response activation. TLR is also a key link between innate and adaptive immune system. Toll-like receptor 4 (TLR4) is mainly expressed in endothelial cells, macrophages, neutrophils, and dendritic cells (DCs). Myeloid differentiation factor 88 (MyD88) is the key adaptation protein of TLR4 (6), which leads to activation of downstream nuclear factor κB (NF-kB) and subsequent production of a series of inflammatory cytokines (7,8). The NF-κB transcription factor has five members in mammals, of which p65 and p50 are most abundant in mammalian cells (9). Gang Li reported that TLR4-mediated NF-κB signaling pathway plays an important role in the development of mouse pancreatic injury (10). As a target, TLR4 controls the order of AP inflammatory response. TLR4 participates in the immune response in the early stage of AP, and eventually induces an inflammatory cascade, leading to deterioration of AP (11). Tim-3 is an important member of the Tim family and plays an important role in CD4$^+$ Th1 cells, T helper cells 17, CD8$^+$ T cells, dendritic cells, natural killer cells, and monocytes/macrophages (12,13). Currently, Tim-3 has been widely recognized as an ideal target for immunotherapy due to its correlations with the pathogenesis of many clinical diseases (14). For example, Tim-3 is a negative regulator of chronic diseases such as human immunodeficiency virus (HIV), hepatitis B virus (15), atherosclerosis (16), and diabetes (17). Tim-3 also plays an important role in maintaining the homeostasis of inflammatory response in sepsis (18,19). In this study, we will explore the possible mechanism of Tim-3-mediated regulation of inflammatory response in the early stage of SAP by establishing a mouse model of SAP, aiming to provide a new target for treatment of AP.

MATERIALS AND METHODS

Experimental Animals. Experimental mouse SAP model is able to closely mimic the main features of human AP (20). In the current study, SAP mouse models were

established in BALB/c mice with intraperitoneal injection of 8% L-arginine. Male specific pathogen free (SPF) BALB/c mice (6-8 weeks old), weighing 20-25 g, were purchased from Experimental Animal Center of Nanjing Medical University. All mice were maintained in the SPF animal experiment center under a condition of 12-hour light/dark cycle. The room temperature was controlled at 22 °C. The rodents had access to drinking water and food as a standard diet. All animal experiments were approved by the Experimental Animal Ethics Committee of Nanjing Medical University.

Animal Model and Experimental Groups. The mice were anesthetized by intraperitoneal injection of sodium pentobarbital with a dose of 100 mg/kg. Animals were fasted for 24 hours before the experiment. After modeling, the mice were still fasted but allowed to drink water. The mice were randomly divided into 3 groups and 15 animals each group. Sham operation group (sham), mice were intraperitoneal injected with sterile saline (400 mg/100 g body weight) twice at an interval of 1 h; SAP group (SAP), mice were intraperitoneal injected with 8% L-arginine twice at an interval of 1 h, the concentration was 400 mg/100 g body weight; Anti-Tim-3 treated group (Anti-Tim-3), down-regulation of Tim-3 was performed by intraperitoneal injection of Tim-3 antibody as described previously (21,22). Mice were intraperitoneal injected with anti-Tim-3 antibody (clone B8.2C12, Bio Cell Co., Shanghai, China) one day before modelling at a dose of 200 μg per mouse. At 24 hours after surgery, mice were sacrificed under anesthesia by intraperitoneal injection of sodium pentobarbital (100 mg/kg).

Histopathological Analysis. Pancreatic tissue specimens were dehydrated, transparent, dipped in wax, embedded, and sectioned. Five high-power fields (×200) were randomly selected for each slice to observe pancreatic tissue changes, and scored according to the pathological scoring criteria described by Schmidt et al (23). The score was as a sum of scores for four parameters of edema, inflammatory infiltration, necrosis and hemorrhage.

Assessment of Myeloperoxidase (MPO) Activity. Neutrophil activity in pancreas was determined by measuring the biological activity of MPO in pancreatic tissues. For these measurements, pancreatic tissues were harvested at the time of mice death and were stored at -80°C. The MPO activity in pancreatic tissue was performed according to the protocol of the detection system (Jiancheng Biological Laboratories Co., Nanjing, China). MPO activity was expressed as units per milligram of protein.

Enzyme-Linked Immunosorbent Assay (ELISA). The serum levels of interleukin-6 (IL-6), IL-10, and tumor necrosis factor-α (TNF-α) were detected by ELISA (Immuno-Biological Laboratories Co., Ltd., Beijing, China) according to the manufacturer's instruction. Serum amylase activity was measured using an Olympus AU2700 automatic biochemical analyzer.

Reverse transcription–PCR (RT-PCR). The isolation and culture of mouse peritoneal macrophages were performed according to the methods described in the literature (24,25). The transcription levels of Tim-3, IL-6, IL-10, TNF-α and TLR4 in mouse peritoneal macrophages were detected by two-step RT-PCR method. Total RNA was extracted from mice peritoneal macrophages with the use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA (1 μg) was reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Cat. NO. 205311, Qiagen, Germany) according to the manufacture's instructions. Real-time PCR was performed in a 20 μL reaction that contained 10 μL of SYBR® Premix Ex Taq™ II, 2 μL of cDNA, 6.8 μL of RNase/DNase-free water and 200nM of each primer pairs. The RT-PCR program was

as follows: initial denaturation at 42°C for 3 min and 95°C for 5 min, followed by 40 cycles of 90°C for 30s and 60°C for 30s, performed on the ABI PRISM 7900 Sequence Detector system (Applied Biosystem, Foster City, CA). Each assay was performed in triplicate. The relative expression of a gene was determined using the comparative Ct method formula $2^{-\Delta\Delta Ct}$, with GAPDH as the internal control. The primer sequences for targeted cDNAs are listed in Table 1.

Table 1. Primers used in this study.

	Sense Primer	Antisense Primer
Tim-3	5'-GACCTCCATAATAACAA-3'	5'-TAATAAGGCTCAAACTCG-3'
IL-6	5'-CCACTTCACAAGTCGGAGGCTTA-3'	5'-GCAAGTGCATCATCGTTGTTCATAC-3'
IL-10	5'-GGGGCCAGTACAGCCGGGAA-3'	5'-CTGGCTGAAGGCAGTCCGCA-3'
TNF-α	5'-ATTCGAGTGACAAGCCTGTAGCCCA-3'	5'-CTGGGAGTAGACAAGGTACAACCCA-3'
TLR4	5'-CACTGTTCTTCTCCTGCCTGAC-3'	5'-CCTGGGGAAAAACTCTGGATA-3'
GAPDH	5'-TCTTGGGCTACACTGAGGAC-3'	5'-CATACCAGGAAATGAGCTTGA-3'

Western Blot Analysis. The total proteins of Tim-3, TLR4, MyD88 and NF-kB p65 were extracted with RIPA Lysis (Thermo Fisher Scientific, CN). Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, CN) was used to detect the concentration of total protein. Proteins (50 µg) were separated by 10%SDS-PAGE, and then electrotransferred to nitrocellulose membranes (Millipore, Bedford, MA). Membranes were incubated overnight together with primary antibodies, including rabbit Abs against Tim-3 (1:1000, Cat. No. 11872-1-AP, Proteintech, USA), TLR4 (1:1000, Cat. No. 19811-1-AP, Proteintech, USA), MyD88 (1:500, Cat. No. 23230-1-AP, Proteintech, USA), p65 (1:500, Cat. No. 10745-1-AP, Proteintech, USA), and β-actin (1:1000, Cat. No. 20536-1-AP, Proteintech, USA). Membranes were then incubated with alkaline phosphatase-conjugated anti-rabbit IgG Abs (1:1000, Cat. No. SA00002-2, Proteintech, USA) and specific bands were detected with a Bio-Rad (Hercules, CA) imaging system. To control for unwanted sources of variation, the relative expression of target proteins was normalized to β-actin. The mean values of the control group were set to 1; the values of other groups were normalized to control group values, presented as fold mean of the controls.

Statistical Analysis. Data were analyzed using Graphpad Prism 5 software (La Jolla, CA, USA) and SPSS 13.0 statistical software (IBM; USA). The data is expressed as mean ± SD. The comparison of multiple groups was performed by randomized block design analysis of variance (ANOVA test), and two groups comparison was performed with t test. For data with non-normal distribution or with unequal variance, two groups were analyzed by Mann-Whitney U test, and multiple groups were analyzed by Kruskal–Wallis test. $p < 0.05$ was considered statistically significant.

RESULTS

Down-regulation of Tim-3 expression aggravated mouse pancreatic tissue damage.
In this study, we first investigated whether the expression of Tim-3 was related to the severity of AP. We successfully established a mouse SAP model by intraperitoneal injection of L-arginine according the method reported previously (20). Compared with the SAP group, the pathological observation in the Anti-Tim-3 treated group showed obvious edema of the pancreatic tissue, a large amount of leukocyte infiltration, extensive necrotic acinar cells, and severe lobular structure disorder (Figure 1a-c). The pancreatic histopathological score (Schmidt criteria) was 1.3 ± 0.5 in the sham group, 6.9 ± 1.0 in the SAP group, and 11.5 ± 1.3 in the Anti-Tim-3 group. Compared with the sham group, the pathological scores of the SAP group and Anti-Tim-3 treated group significantly increased ($p<0.05$) while the pathological score of the Anti-Tim-3 treated group was significantly higher than the SAP group ($p<0.05$) (Figure 1d). The degree of inflammatory cell infiltration in pancreatic tissues could be determined through detecting the MPO activity in pancreatic tissues. The results showed that compared with the sham group (1.8 ± 0.1 unit/g protein), the MPO activity of the pancreatic tissues in the SAP group (3.1 ± 0.5 unit/g protein) and Anti-Tim-3 treated group (5.4 ± 0.6 unit/g protein) significantly increased ($p<0.05$), while the MPO activity of the pancreatic tissue in the Anti-Tim-3 treated group was significantly higher than that in the SAP group ($p<0.05$) (Figure 1e).
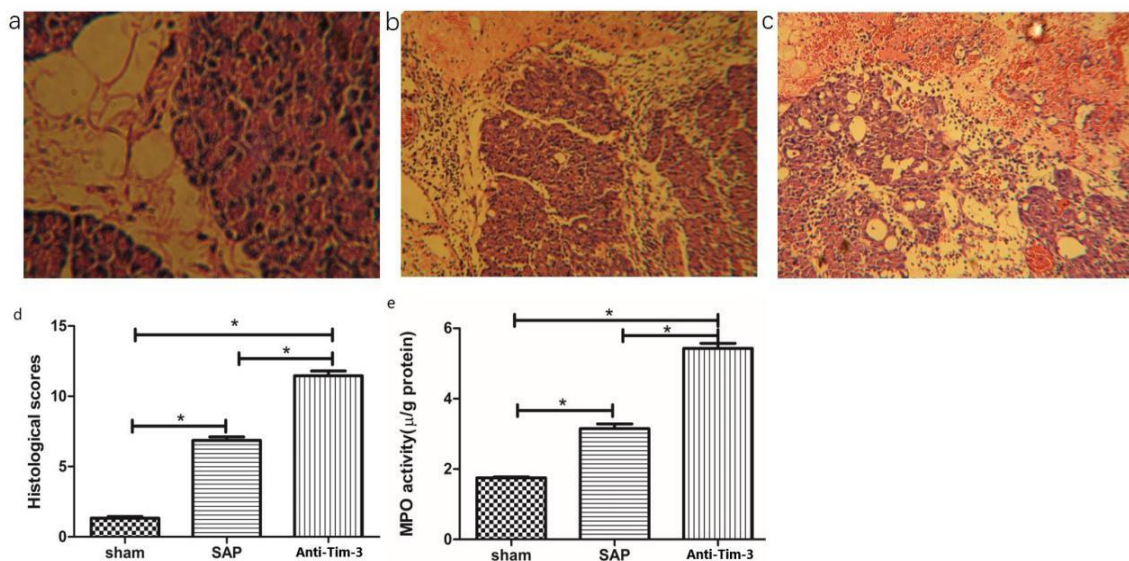

Figure 1. Histopathological and MPO changes of the pancreas. Histopathological changes of the pancreas: representative H&E-stained micrographs (original magnification, x200) from the (a) sham, (b) SAP and (c) Anti-TIM-3 treated groups are presented. The pancreatic tissue inAnti-Tim-3 treated group exhibited marked edema and infiltrated by leukocyte; a mass of necrotic acinar cells and the severe loss of lobules were also observed. (d) Histopathological scores of the pancreas in the sham, SAP and Anti-TIM-3 treated groups. (e) Results of MPO: MPO was significantly higher in Anti-Tim-3 treated group than SAP and sham group. Data are expressed as the mean ± SD (n=15/group). Sham, the sham injection group; SAP, the SAP Group; Anti-Tim-3, the Anti-Tim-3 treated group. *; p<0.05.

Downregulation of Tim-3 expression promoted the production of pro-inflammatory cytokines IL-6 and TNF-α in peripheral blood of mice.
Using ELISA, we examined the production of pro-inflammatory cytokines (IL-6, TNF-α) and anti-inflammatory cytokines (IL-10) in peripheral blood. The results showed that the expression of pro-inflammatory cytokines IL-6 (794 ± 141 pg/ml) and TNF-α (440 ± 100 pg/ml) significantly increased in Anti-Tim-3 treated group compared with the sham group (89 ± 5 pg/ml, 42 ± 23 pg/ml) ($p<0.05$). In addition, the results showed that the expression of anti-inflammatory cytokine IL-10 significantly increased in Anti-Tim-3 treated group (187 ± 70 pg/ml) compared with the sham group (58 ± 23 pg/ml) ($p<0.05$) (Figure 2). However, the expression level of the anti-inflammatory cytokine IL-10 in the peripheral blood of Anti-Tim-3 treated group (187 ± 70 pg/ml) decreased compared with the SAP group (251 ± 104 pg/ml) but the difference was not statistically significant (Figure 2c).
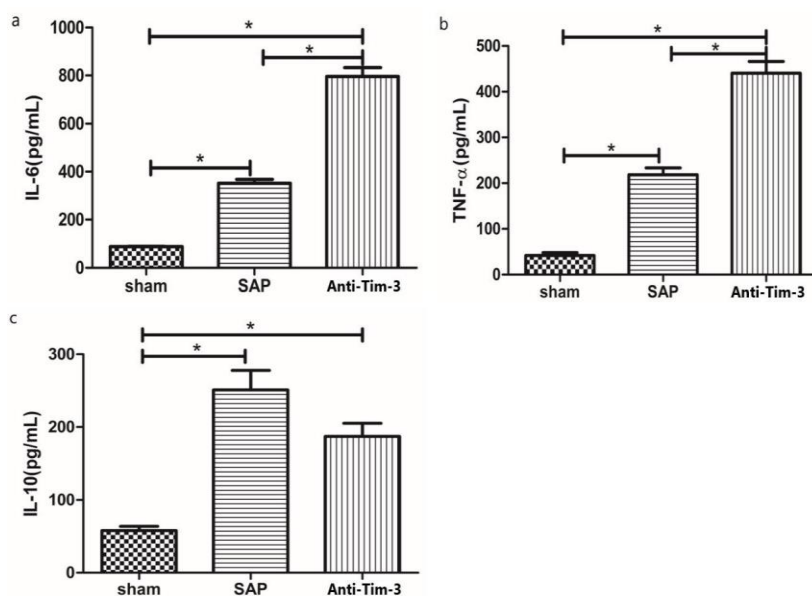

Figure 2. Pro- and anti-inflammatory cytokine expression in the peripheral blood. (a) IL-6 expression, compared to the sham group, IL-6 were significantly elevated in the SAP and Anti-Tim-3 treated group. (b) TNF-α expression, compared to the sham group, TNF-α were significantly elevated in the SAP and Anti-Tim-3 group. (c) IL-10 expression, compared to the sham group, TNF-α were significantly elevated in the SAP and Anti-Tim-3 group; compared to the SAP group, IL-10 were increased in the Anti-Tim-3 group. Data are expressed as the mean ± SD (n=15/group). Sham, the sham injection group; SAP, the SAP Group; Anti-Tim-3, the Anti-Tim-3 treated group. *; p<0.05

Regulation of inflammatory factors in mouse peritoneal macrophage by Tim-3 expression.
Our results suggested that the down-regulation of Tim-3 expression aggravated the inflammatory response in peripheral blood in vitro. The relationship between peritoneal macrophages and systemic inflammatory response induced by AP has been the focus of research on the pathogenesis of AP (26). We further studied the immunomodulatory

effects of Tim-3 in peritoneal macrophages. The PCR and western blot analysis confirmed that the mRNA and protein levels of Tim-3 in peritoneal macrophages were significantly reduced following the intraperitoneal injection of Tim-3 specific antibody (Figure 3a, Figure 4a, 4b). Furthermore, the downregulation of Tim-3 significantly affected the production of inflammatory cytokines in peritoneal macrophages. Compared with the sham group (0.9 ± 0.1) and the SAP group (2.0 ± 0.2), the mRNA level of IL-6 significantly increased in Anti-Tim-3 treated group (3.6 ± 0.3). Additionally, compared with the sham group (1.0 ± 0.1) and the SAP group (1.5 ± 0.1), the mRNA level of TNF-α significantly increased in Anti-Tim-3 treated group (2.2 ± 0.3) (Figure 3b, 3c). The mRNA level of anti-inflammatory factor IL-10 in peritoneal macrophages of the SAP group (1.6 ± 0.2) and Anti-TIM-3 treated group (1.6 ± 0.1) obviously increased in comparison with the sham group (p<0.05).
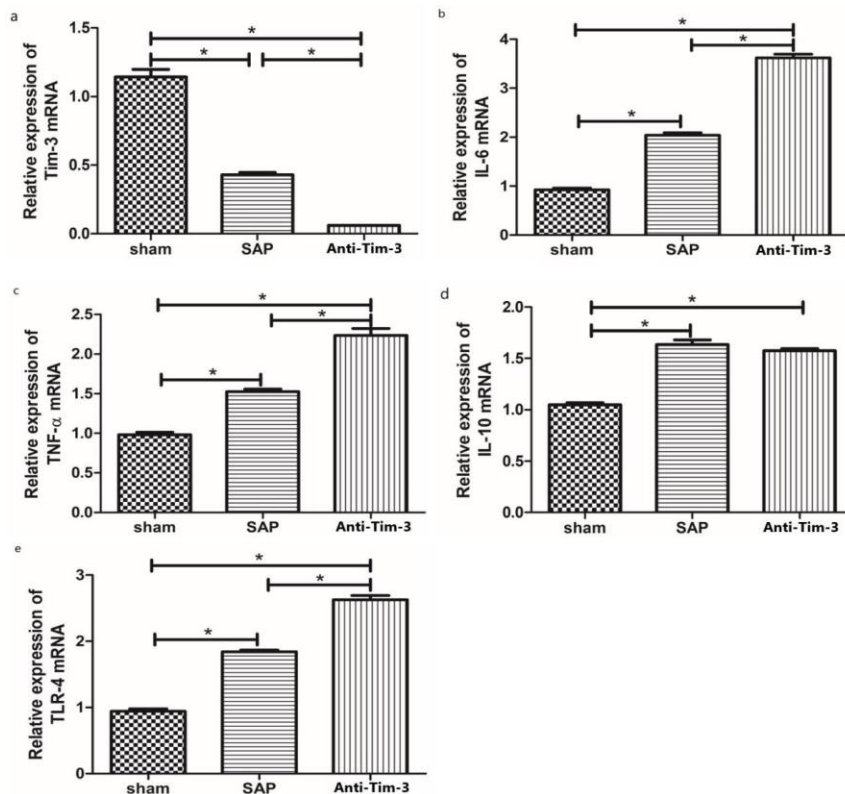

Figure 3. Pro- and anti-inflammatory cytokine in peritoneal macrophage. (a) Tim-3 mRNA expression, the expression of Tim-3 mRNA in peritoneal macrophage was significantly reduced after intraperitoneal injection of anti-Tim-3 mAb. (b) IL-6 mRNA expression, (c) TNF-α mRNA expression, compared to the sham and SAP group, the expressions of mRNA for pro-infalmmatory cytokines IL-6 and TNF-α were all significantly upregulated in macrophage that had received anti-Tim-3 mAb (Anti-Tim-3 group). (d) IL-10 mRNA expression, compared to the SAP group, the expression of mRNA for anti-infalmmatory cytokine IL-10 was no obvious changed in the Anti-Tim-3 group. (e) TLR4 mRNA expression, compared to the sham group, the expression of TLR4 mRNA was significantly increased in the SAP and Anti-Tim-3 group; compared to the SAP group, the expression of TLR4 mRNA was significantly increased in the Anti-Tim-3 group. Data are expressed as the mean ± SD (n=15/group). sham, the sham injection group; SAP, the SAP Group; Anti-Tim-3, the Anti-Tim-3 treated group. *; p<0.05

Nevertheless, there was no significant difference between the SAP group and Anti-TIM-3 treated group concerning the expression level of IL-10 in peritoneal macrophages (Figure 3d). TLR4 plays a key role in the inflammatory response of AP; therefore, we also examined the mRNA expression of TLR4 in peritoneal macrophages. The results showed that the expression level of TLR4 mRNA in peritoneal macrophages of the SAP group (1.8 ± 0.1) and Anti-TIM-3 treated group (2.6 ± 0.3) was significantly higher than the sham group (0.9 ± 0.1) ($p<0.05$). In addition, compared with the SAP group, the expression level of TLR4 mRNA in the peritoneal macrophages of Anti-Tim-3 treated group significantly increased (Figure 3e).

The release of inflammatory cytokines in peritoneal macrophages was regulated by Tim-3 through TLR4-MyD88-NF-κB pathway.

Our findings suggested that Tim-3 regulated the production of inflammatory cytokines and TLR-mediated signaling pathway in peritoneal macrophages. Since the TLR4/MyD88 pathway plays a key role in TLR4-induced inflammatory cytokine production, we further determined whether down-regulation of Tim-3 can affect the activity of TLR4, MyD88, and NK-κB. NF-κB is a family of transcription factor proteins, of which p65 is the most abundant in cells (10), so we evaluated the activity of NK-κB by detecting the activity of p65 factor. The results showed that compared with the SAP group, the protein expression of TLR4 (0.9 ± 0.1), MyD88 (0.7 ± 0.03) and p65 (0.9 ± 0.05) in the peritoneal macrophages of the Anti-Tim-3 treated group was significantly increased (Figure 4). This suggests that the downregulation of Tim-3 expression in peritoneal macrophages promoted the activation of TLR4/MyD88 and NF-κB. Although the mechanisms involved in this process are still unclear, our results indicated that Tim-3 might modulate the release of inflammatory cytokines through negatively regulating the TLR4-MyD88- NF-κB signaling pathway in peritoneal macrophages.

DISCUSSION

In the present study, we demonstrated for the first time that peritoneal macrophage surface protein Tim-3 was involved in the early regulation of AP. It was also found that down-regulation of Tim-3 expression aggravated pancreatic tissue damage, and its mechanism might be related to the inhibition of inflammatory factor release by Tim-3 through negative regulation of TLR4-MyD88-NF-κB signaling pathway. AP is an inflammatory disease and which is classified as mild AP (MAP) and severe AP (SAP) according to mild or severe local/systemic inflammatory responses. The initial local lesion is characterized by local damage and necrosis of pancreatic acinar cells induced by damage-associated molecular pattern (DAMP). MAP usually declines during the first week of onset, whereas SAP is characterized by a more severe systemic immune response and develops into multiple organ dysfunction that lasts for more than 48 hours (27). In the early stages of SAP, SIRS can further cause MOF, resulting in a significantly increased mortality (28). A large number of inflammatory cells and molecules are involved in the development of AP from local inflammation to MAP or SAP (29). In this study, by employing a mouse SAP model, obvious local damage and inflammatory response of the pancreas were observed, and a large amount of inflammatory factors were also released from peripheral blood.
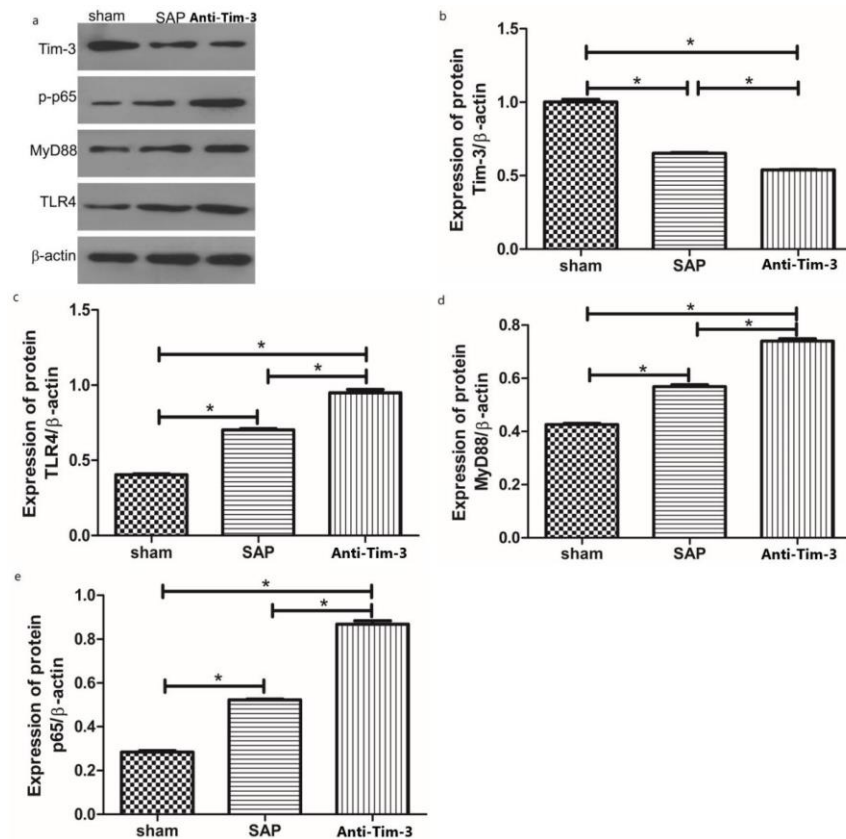
Figure 4. Tim-3 blockade increased TLR4, MyD88 and p65 expression in peritoneal macrophage. (a) Western blot analysis using Abs against Tim-3, TLR4, MyD88, p-p65 and β-actin. (b) Tim-3 was stable blockaded in Tim-3 group. (c-e) Tim-3 blockade significantly increased TLR4, MyD88 and p-p65 expression, compared with the SAP group. Data are expressed as the mean ± SD (n=15/group). sham, the sham injection group; SAP, the SAP Group; Anti-Tim-3, the Anti-Tim-3 treated group. *;p<0.05.

Macrophages are important immune cells in the body and play an important role in inflammation and tumor immunity. Primary mononuclear cells are released from the bone marrow and migrate into tissues, and eventually differentiate into specific types of macrophages. The macrophages in these tissues vary in phenotype, including Kupffer cells in the liver, alveolar macrophages in the lungs, osteoclasts in the bone, microglia in the brain, Langerhans cells in the skin, etc. (30). Abdomen is adjacent to the necrotic site of pancreatitis, which is the origin of inflammation. The released IL-6 and TNF-α from peritoneal macrophages in early AP can induce the cascade of other inflammatory cytokines, neutrophil activation and systemic inflammatory response. Therefore, peritoneal macrophages in AP are the main initiators of acute systemic inflammatory response, which determines the progression and severity of the disease (31,32), while blocking the release of inflammatory factors from peritoneal macrophages may inhibit systemic inflammatory response and reduce mortality. Consistently, the present study also found that a large number of pro-inflammatory factors IL-6 and TNF-α, as well as anti-inflammatory cytokine IL-10 were produced in the peritoneal macrophages of the SAP group. Abnormal inflammatory response plays an important role in the occurrence and development of AP. In the early stage, excessive inflammatory responses control

the pathophysiological process, leading to SIRS and MOF (3). Therefore, how to control the excessive inflammatory response of peritoneal macrophages at the early stage of AP is very important. Tim-3 is an important member of the T cell immunoglobulin and mucin domain (Tim) family and is involved in immune regulation. Recent studies have found that at the early stage of bacterial and viral infections, the expression of Tim-3 on the surface of T cells and macrophages is elevated, and its expression level is even higher in pathogenic microorganism-specific immune cells. Frisancho-Kiss *et al.* (33) reported that Tim-3 was involved in the immune regulation of early inflammatory responses in the body and might act as a macrophage activation inhibitor. Yang Xiaomei *et al.* (18) observed that the upregulation of Tim-3 on the surface of mononuclear macrophages from sepsis was related to lipopolysaccharide (LPS) stimulation, and further demonstrated from in vitro and in vivo that blockage or downregulation of Tim-3 expression could enhance macrophage activity. More and more research has begun to focus on the role of TIM-3 in various diseases as a new immune checkpoint molecule that regulates the strength or duration of immune responses (34). Therefore, Tim-3 is a potential target for immune intervention against infections. In this study, the data indicated that Tim-3 mRNA and Tim-3 protein expression in peritoneal macrophage were significantly reduced after intraperitoneal injection of anti-Tim-3 antibody. Several possible mechanisms may explain our finding: (1) anti-Tim-3 antibody may cross-link Tim-3 protein on the surface of macrophage in vivo; (2) anti-Tim-3 antibody could block interaction of Tim-3 with its potential ligand on macrophages (21). Furthermore, we observed its immunomodulatory effects in the early stage of AP and possible mechanisms. The results showed that after down-regulating the expression of Tim-3, the production of inflammatory factors in peritoneal macrophages was significantly increased, and the inflammatory reaction in peripheral blood was aggravated, resulting in aggravation of pancreatic tissue damage in mice. These results indicated that peritoneal macrophage Tim-3 maintains the balance of inflammation in the early stages of AP by negatively regulating the inflammatory response.

How does peritoneal macrophage Tim-3 negatively regulate the inflammatory response? The TLR4 signaling pathway plays an important role in the AP. It has been found that TLR4-mediated NF-κB signaling pathway plays an important role in the development of pancreatic injury in mice (10). When TLR4 binds to pathogen-associated molecular pattern (PAMP) or DAMP, the intracellular NF-κB signaling pathway is activated, completing the signal transduction process of inflammation (35,11). Many studies have focused on the different targets of TLR4 signaling pathway to reach the therapeutic purposes. For example, blocking the binding of TLR4 to PAMP/DAMP, inactivation of TLR4 by altering its configuration, or inhibiting excessive activation of related factors in the TLR4 signaling pathway (36,37). In the present study, we focused on the MyD88-dependent signaling pathway. It was found that the protein expression of TLR4, MyD88 and p65 in peritoneal macrophages was significantly increased in the early stage of mouse SAP. Interestingly, the increase of TLR4, MyD88 and p65 expression in peritoneal macrophages was more significant when the expression of Tim-3 on the surface of peritoneal macrophages was down-regulated. Therefore, it is very possible that Tim-3 on peritoneal macrophages may regulate the release of inflammatory cytokines by immunoregulating the expression of TLR4, MyD88 and p65 proteins in the TLR4/MyD88-NF-κB signaling pathway, thereby affecting inflammatory response in AP. There are several limitations in this study. First, it is still unclear whether the

inflammatory response of AP can be alleviated by downregulating the TLR4, MyD88 and p65 expression in TLR4/MyD88-NF-κB signaling pathway through upregulation of Tim-3 on the surface of peritoneal macrophages. We have successfully constructed a eukaryotic expression vector pTARGET-Tim-3, future study will try to address this question. Secondly, it is still unclear how Tim-3 regulate proteins TLR4, MyD88 and p65, which needs to be further studied. In summary, our study demonstrate that Tim-3 may play an important role in the development of AP by regulating the inflammatory response. However, further studies are required to investigate the mechanism of Tim-3-mediated regulation of inflammatory response in AP, as well as the possibility of using Tim-3 as a target for immunotherapy of AP.

ACKNOWLEDGEMENTS

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