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Down-Regulation of Tumor Necrosis Factor-Associated Factor 6 Is Associated with Progression of Acute Pancreatitis Complicating Lung Injury in Mice

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Acute lung injury is one of the critical complications of acute pancreatitis (AP). Tumor necrosis factor-associated factor 6 (TRAF6) is a key adaptor that regulates various inflammatory signaling pathways, including those mediated by Toll-like receptors (TLRs). This study was performed to investigate the potential role of TRAF6 in the pathogenesis of AP and pancreatitis-associated acute lung injury using a mouse model of caerulein-induced AP (CAP). CAP was induced by intraperitoneal injection of caerulein hourly for 7 times (50 μg/kg), and control mice were treated with saline of the same volume. Typical pancreatic and lung inflammation was observed in the early stage (1 h) of CAP, as judged by morphological changes. Likewise, in CAP mice, the pancreatic myeloperoxidase activity and serum levels of interleukin-6 and interleukin-10 were significantly increased after 2 h, peaked at 4h, and then decreased by 24 h. The expression of TRAF6 was then studied by real time-PCR, immunohistochemistry, and Western blot analysis. Compared with control group, TRAF6 mRNA level was decreased in CAP group within the first 12 h, and then significantly increased after 24 h, which was in accordance with the protein level detected by Western blot analysis and immunohistochemistry. Moreover, TRAF6 protein was expressed in both pancreatic acinar cells and lung bronchial epithelial cells. In conclusion, the down-regulation of TRAF6 was associated with increased inflammatory severity in the pancreas and lung, suggesting that TRAF6 is involved in the anti-inflammatory process during AP. TRAF6 may be a potential molecular target for treating AP. ——— inflammation; pathogenesis; acute pancreatitis; tumor necrosis factor-associated factor 6; acute lung injury.

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Acute pancreatitis (AP) is a common disease with great variability in severity. In mild cases, the process of AP is self-limited, while its severe form is characterized by pancreatic necrosis and inflammatory cytokine activation, which will lead to multiple organ dysfunction (Lupia et al. 2004; Ohashi et al. 2006). Therefore, investigating the mechanism of inflammation cascade reactions during AP is of great value for clinical therapy.

Innate immunity is the first step of host defense against inflammatory stimuli. Toll-like receptors (TLRs) as the central receptor of innate immunity have become focus of studies on inflammatory mechanism (Cao et al. 2007; Yang et al. 2008). After recognizing various ligands, TLRs transduce extracellular signal into cells, induce the activation of nuclear factor-κB (NF-κB) and activating protein-1 (AP-1), and then result in inflammatory cascade reactions (Sekine et al. 2006).

In most of the TLRs-mediated signaling pathways, tumor necrosis factor-associated factor 6 (TRAF6) is the central convergence (Kawai and Akira 2005). Accumulating evidence has demonstrated that TRAF6 regulates various inflammation processes by distinct mechanism (Wu and Arron 2003). TLRs activate inflammatory and apoptotic signaling pathways via TRAF6. The lack of TRAF6 may lead to signaling defection, NF-κB inactivation and reduction of cytokine production induced by TLRs ligand (Gohda et al. 2004). Many biological effects induced by TRAF6 are generated through activating the IκB kinase complex and the members of mitogen-activated protein kinases (Loniewski et al. 2007).

Recent investigations demonstrated that TLRs participate in AP and pancreatitis-associated lung injury (Geisler et al. 2005; Wang et al. 2005; Matsuda et al. 2006; Zeng et al. 2008). However, TLRs downstream signaling events

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that regulate innate immunity in response to inflammation have not been well investigated. Therefore, we hypothesize that TRAF6, the important TLRs signal adaptor, is responsible for the pathogenesis of AP. The aim of this study was to clarify the role of TRAF6 in pancreatic and lung inflammatory response in experimental AP.

Material and Methods

Animals, grouping, AP inducing and tissue sampling

Male C57BL/10SnJ mice of 22-24g in weight, aged 6-8 weeks, were used in this study. These mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in the Experimental Animal Center of Sichuan University (Chengdu, China). Mice were housed in a controlled environment and fed with standard rodent chow and water.

The mice were divided into 2 groups randomly: caerulein-induced acute pancreatitis (CAP) group and saline-treated control group. CAP group was further divided into 5 subgroups depending on the time of sampling: 1, 2, 4, 12, and 24 h (n = 8 for each time point). Saline-treated control was set for each experimental group. All the animals were maintained at 23°C on a 12 hours light/dark cycle and starved for 12 hours prior to experimentation except water. The study was approved by the Animal Ethics committee of Sichuan University West China Medical School, and all procedures with animals were conducted according to the guidelines of the local Animal Use and Care Committees of Chengdu and executed according to the National Animal Welfare Law of China.

AP was induced by intraperitoneal injection of caerulein (Sigma, St. Louis, MO, USA) at a dosage of 50 μg/kg body weight. Caerulein was hourly injected for 7 times, and the control mice were treated with the saline of the same volume for 7 times, respectively. After the last injection, sampling was made at 1, 2, 4, 12, and 24 h.

Under 1% pentobarbital anesthesia (50 mg/kg body weight), a laparotomy was performed. Fresh pancreatic tissues and lung tissues were removed, pancreatic tissues around the pancreatic duct were rinsed in Trizol (Gibco, Carlsbad, CA, USA) for subsequent RNA isolation, and other pancreatic tissues and lung tissues were frozen at –80°C for further studies. Inferior vena cava blood was collected and centrifuged at 4°C, and the serum was taken for further studies.

Histological examination

For routine histology, 4-μm sections of 10% formalin-fixed, paraffin-embedded tissue were prepared and stained with hematoxylin and eosin (HE). All microscopic sections were analyzed in a blind fashion.

Myeloperoxidase (MPO) assay

Neutrophil sequestration in pancreas was quantitated by measuring tissue MPO activity. MPO was detected according to the protocol of the Chrrmatometric kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, after weighed, the pancreatic samples were thawed, homogenized in 1 ml of 20 mM phosphate buffer (pH 7.4), centrifuged (10,000 × g, 10 min, 4°C), and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltmethyl-ammonium bromide. The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (40 s). The sample was then centrifuged (10,000 × g, 5 min, 4°C) and the supernatant was used for MPO assay by utilizing 0.0005% hydrogen peroxide as a substrate. MPO activity was determined by degrading 1 μmol of peroxide per min at 25°C. Results were expressed as units per g weight (U/g) of wet tissue.

LiquiChip multiple cytokines assay

Serum cytokines, interleukin (IL)-6 and IL-10, were simultaneously measured on LiquiChip-100 workstation (Luminex, Austin, TX, USA) by using LiquiChip cytokine kits (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

Quantitative real-time reverse transcription-polymerase chain reaction (real time RT-PCR)

Fresh pancreatic tissue (50 mg) was collected per mouse, and total RNA was extracted with TRIzol (Gibco, Carlsbad, CA, USA). RNA was reverse-transcribed, and the cDNA was subjected to PCR. Specific primers and Taqman probe of TRAF6 were designed and synthesized by TaKaRa Co. (Dalian, China), the sequences of primer and probe sets were listed in Table 1. Conditions for all PCRs were optimized on iCycler iQ (Bio-Rad, Hercules, CA, USA) in a 30-μl reaction system. The following program was run for 40 cycles: 94°C for 20 s, 51°C for 30 s and 72°C for 30 s. All samples were amplified simultaneously in triplicate in one assay-run. The amplified products were resolved by electrophoresis in 2.0% agarose gels and visualized by gold view (Sai Bai Sheng, Bei Jing, China) staining.

Immunohistochemistry (IHC)

Briefly, 5-μm sections of paraffin-embedded sections were deparaffinized in xylene, rinsed in ethanol and rehydrated. Antigen retrieval was performed by microwave for 12 min in TE buffer. After being washed in PBS and treated with 0.3% H_2O_2 for 30 min in the dark, the sections were blocked with a 1:20 dilution of rabbit serum albumin for 30 min; Then, the sections were incubated with 1 : 200 dilution of goat anti-mouse TRAF6 monoclonal antibody (Upstate, Charlottesville, VA, USA) overnight at 4°C, incubated with a 1 : 200

Table 1. Specific primers and probes for tumor necrosis factor- associated factor 6 (TRAF6) cDNA.

Gene	Primer	length
TRAF6	F: 5´-TCTGCTTGATGGCTTTACG-3´ R: 5´-ACCGTCAGGGAAAGAATCT-3´ Probe: 5´-AGCAGTGCAAACACCATGTGGC-ECLIPSE-3´	181
β-actin	F: 5´-CGTGAAAAGATGACCCAGAT-3´ R: 5´- ACCCTCATAGATGGGCACA-3´ Probe: 5´-TCAACACCCCAGCCATGTACGT-TAMRA-3´	158

dilution of biotinylated rabbit-anti-goat IgG (Zhongshan Biotech, Beijing, China) for 30 min and 1 : 200 dilution of Horseradish peroxidase-avidin complex (Zhongshan Biotech, Beijing, China) incubated for 30 min sequentially. After washing the sections with PBS for three times, the tissue was visualized by reaction in a solution containing 1 : 50 3,3´-diaminobenzidine (DAB) in 0.05% H_2O_2. Finally, the sections were washed, counterstained with hematoxylin, dehydrated, and sealed. IHC score was applied to semi-quantitatively evaluate TRAF6 expression in each section. The total score was based on staining intensity (0–6) and positive cell number (0: ≤10%; 1: 11–25%; 2: 26–50%; and 3: >50%). The total score ≤2 was considered low/negative expression (±); 3–4: moderate expression (++); 5–6: intensive expression (+++).

Western blot analysis

Pancreas and lung tissues were homogenized with lysate buffer, treated with SDS-polyacrylamide loading buffer at 95°C for 5 min, separated by 10% SDS-polyacrylamide gel electrophoresis, transferred from the gels into a polyvinilidine difluoride membrane (PVDF, Bio-Rad, Hercules, CA, USA), and incubated with a primary antibody in a 1 : 500 dilution goat anti-mice TRAF6 monoclonal antibody (Upstate, Charlottesville, VA, USA) in TBST for 2 h at room temperature. After TBST washing, the membranes were incubated with 1 : 5000 dilution peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) or a monoclonal anti-β-actin for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system (Pierce Biotechnology, Rockford, IL, USA), and the semi-quantitative analysis of grayscale intensity was generated with Multi Gauge V3.o software (Fujifilm, Tokyo, Japan).

Statistical analysis

The data were expressed as mean ± S.E.M. Differences between three or more groups were evaluated by one-way ANOVA. Student's *t*-test was used for comparison of two groups. A *p*-value of less than 0.05 was considered statistically significant. The tests were performed using the statistical package SPSS software 11.5 (SPSS Inc, Chicago, IL, USA).

Results

Histological findings

Histological findings confirmed that typical AP and pancreatitis-associated acute lung injury were successfully generated in our mouse model. The interstitial edema, acinar vacuolization, inflammatory cell infiltration and pancreatic necrosis were observed in pancreatic tissues of CAP mice (Fig. 1B). The presence of interstitial edema, infiltration of inflammatory cells and thickened alveolar walls were observed in lung tissues of CAP mice (Fig. 1D). There were no histological changes in the pancreas and lung tissues of control mice (Fig. 1A and Fig. 1C).

Increased pancreatic MPO activity and serum cytokines during early phase of CAP

Neutrophil infiltration was assessed by measuring the MPO activity. Pancreatic MPO activity was significantly increased in CAP mice at the 1, 2 and 4 h when compared with respective control mice (control 1 h and control 4 h), followed by the subsequent decrease (Table 2). Control 1 h and control 4 h represent that the pancreas and serum samples was prepared at 1 h and 4 h after saline injection, respectively.

Moreover, serum cytokines of IL-6 and IL-10 were measured. Compared with control 1 h and control 4 h, IL-6

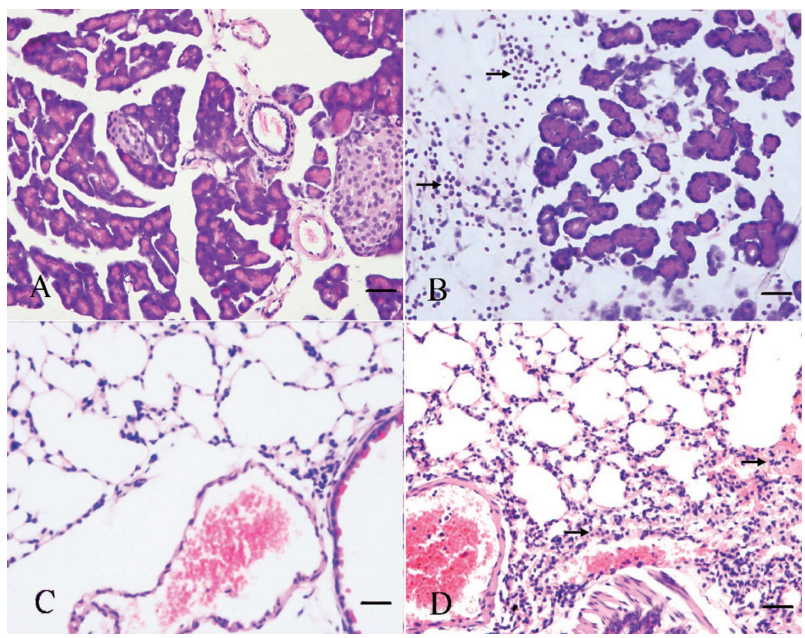

Fig. 1. Histological examination of pancreas and lung; Hematoxylin and eosin (H-E) staining.
A and C, representative pancreas and lung sections of 1 h saline control mice (A, pancreas; C, lung). B, representative pancreas sections of 1 h CAP mice. The arrows represent infiltration of neutrophils. D, representative lung sections of 1 h CAP mice. The arrows represent infiltration of neutrophils. Bar length equals 5 μm. (magnification ×400)

and IL-10 levels were both increased but at different time courses in CAP mice. In CAP mice, IL-6 was significantly increased at 2 h, peaked at 4 h, and then returned to baseline level; IL-10 level was also increased and showed a similar trend as that of IL-6, which peaked at 4 h (Table 3).

Decreased expression of TRAF6 mRNA during acute inflammation

Expression of TRAF6 mRNA in the pancreatic tissue was examined by quantitative RT-PCR. Compared with the 1 h saline-treated control mice, TRAF6 mRNA level in CAP mice was decreased rapidly at 1 h, then began to increase, and peaked at 24 h (Fig. 2).

Decreased expression of TRAF6 protein during acute inflammation

IHC was used to detect and locate the expression and distribution of TRAF6 in pancreas and lung. In pancreatic sections, intensive TRAF6 staining was principally distributed in cytoplasm of the pancreatic acinar cells, weaker staining was also detected in endocrine islets, and no positive staining was detected at the pancreatic duct and vascular system (Fig. 3A-C). In lung sections, intensive TRAF6 staining was mainly distributed in cytoplasm in the lung bronchial epithelial cells, and weaker staining in alveolar cells (Fig. 3D-F).

IHC scoring revealed that TRAF6 expression was significantly lower in CAP mice during the early stage (1, 2, 4 and 12 h) than control mice at 1 h after saline injection, but increased at 24 h (Fig. 4A). Likewise, the change in TRAF6 expression in lung was almost in parallel to that in pancreas (Fig. 4B).

Finally, the change in the TRAF6 protein expression levels in pancreas and lung was evaluated by Western blot

TABLE 2. Myeloperoxidase (MPO) activity in the pancreas tissue (U/mg, mean ± S.E.M.).

Group	Case	MPO
Con 1 h	6	222.00 ± 10.83
Con 4 h	6	231.13 ± 9.76
1 h	6	477.83 ± 30.24*#
2 h	6	797.50 ± 34.25*#
4 h	6	1255.50 ± 126.66*#
12 h	6	608.17 ± 30.27*#
24 h	6	230.50 ± 17.69

Con 1 h and Con 4 h represent the values at 1 and 4 h after saline injection.

*$P < 0.05$ vs Con 1 h; #$P < 0.05$ vs Con 4 h.

TABLE 3. Interleukin (IL)-6 and IL-10 of serum cytokine level (pg/ml, mean ± S.E.M.).

Group	Case	IL-6	IL-10
Con 1 h	8	15.21 ± 0.84	7.56 ± 0.82
Con 4 h	8	14.34 ± 1.21	8.23 ± 0.69
1 h	8	38.36 ± 7.91	15.37 ± 2.12*#
2 h	8	56.28 ± 3.19*#	14.64 ± 3.82*#
4 h	8	182.38 ± 24.25*#	28.52 ± 3.27*#
12 h	8	45.37 ± 11.39*#	14.59 ± 1.62*#
24 h	8	26.65 ± 3.14	11.21 ± 0.94

Con 1 h and Con 4 h represent the values at 1 h and 4 h after saline injection.

*$P < 0.05$ vs Con 1h; # $P < 0.05$ vs Con 4 h.

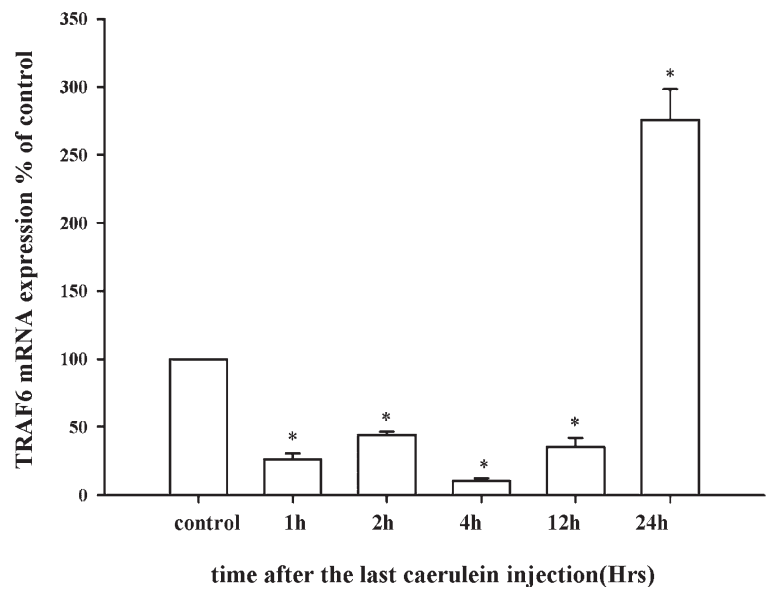

Fig. 2. PCR quantification of TRAF6 mRNA expression in pancreas.
Quantitative PCR was carried out to determine pancreatic mRNA levels. Data were expressed as percent of control at 1 h after saline injection. Results were expressed as mean ± S.E.M ($n = 8$); * $p < 0.05$ vs control. Control represents at 1 h after the completion of saline injection.

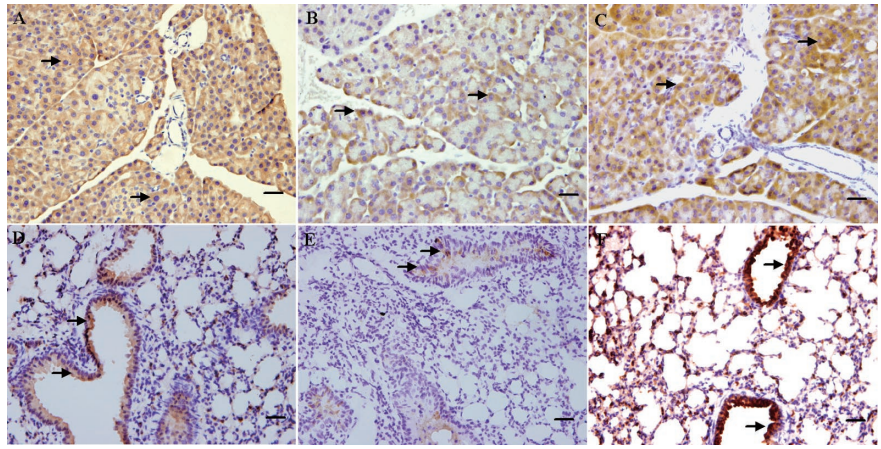

Fig. 3. Immunohistochemical localization of TRAF6 protein in pancreas and lung.
A and D, intense TRAF6 staining was detected in control mice at 1 h after saline injection (A, pancreas; D, lung). The tissues of CAP mice were obtained at 1, 2, 4, 12, and 24 h after caerulein administration: B and E, representative sections at 4 h (B, pancreas; E, lung). C and F, representative sections at 24 h (C, pancreas; F, lung). In pancreas sections, intensive TRAF6 staining was principally distributed in cytoplasm of the pancreatic acinar cells (arrows), weaker staining for TRAF6 also was detected in endocrine islets. In lung sections, intensive TRAF6 staining was mainly distributed in cytoplasm of the lung bronchial epithelial cells (arrows), weaker staining could also be found in alveolar cells. Control represents the value at 1 h after saline injection. Bar length equals 5 μm. (magnification ×400)

analysis (Fig. 5A). Compared with the 1 h saline-treated mice, the expression level of TRAF6 protein was significantly decreased by 1 h, maintained at the low level at 2 h and 4 h, and then increased at 12 h and 24 h in the pancreas and the lung in CAP mice (Fig. 5B).

Discussion

AP is triggered by aberrant premature activation of digestive enzymes within pancreatic acinar and subsequent autodigestion of the pancreas, but the pathogenesis of inflammatory reaction in AP is only partly understood. Therefore, it is important to investigate the exact molecular mechanism of cascade reactions that resulted in pancreatic inflammation.

In the present study, we investigated the role of TRAF6 in the mouse model of CAP. Typical pathological changes of AP and pancreatitis-associated acute lung injury were observed in CAP mice, as judged by histology, MPO as well as circulating cytokines. Our results demonstrated that the severity of inflammation of pancreas and lung increased obviously in the early stage of AP, then gradually restored to the basal level.

In order to investigate whether TRAF6 is associated with inflammatory process during AP, we applied real-time RT-PCR to quantitatively analyze pancreatic TRAF6 mRNA level. The results clearly showed that, in comparison to control mice, TRAF6 mRNA level in CAP mice was decreased in the early AP (within 12 h), and increased in a delayed manner, with peak levels detected at 24 h. The results suggested that TRAF6 participated in the pathogenesis of CAP.

TRAF6 is a key signal adaptor that medicates diverse TLRs, and may exert pro-inflammatory and apoptotic effects (Yeiser et al. 2004). Recently, evidence has accumulated showing the significance of TRAF6 in various inflammatory processes, such as lung injury (Cho et al. 2005), *H. pylori* infection-induced gastric diseases (Hirata et al. 2006), and hepatic inflammation (Slotta et al. 2006). Most of the above-mentioned studies showed that TRAF6 expression was up-regulated in various tissues, implying its pro-inflammatory function. Interestingly, Karimi et al. (2006) found that in cigarette smoke-induced lung inflammatory reaction, cigarette smoke caused subsequently degradation of TRAF6, and triggered the recruitment of neutrophils. Some researches showed during sepsis, activation of TRAF6 might mediate several apoptosis-related signaling pathways, which may be a protective strategy in inflammation processes (Hull et al. 2002; Wong et al. 2004). But the exact function of TRAF6 in AP still remains unclear. Our result showed that down-regulation of TRAF6 was correlated to an increased inflammatory severity; *vice versa,* up-regulation of TRAF6 was linked to palliative pancreatic inflammation. With regard to the inconsistent changing trend between TRAF6 expression and inflammatory severity, one possible explanation is that TRAF6 may exert protective role by mediating apoptotic signaling. Further investigation is required to clarify the exact mechanism.

The pancreatic acinar cells are the functional units of the exocrine pancreas, and the pathophysiology of AP involves dysfunction of pancreatic acinar cells (Leung and Ip 2006). Several studies suggested that the acinar cell response to injury might be an important determinant of disease severity. Mild AP was found to be associated with extensive apoptotic acinar cell death, while severe AP was found to involve extensive acinar cell necrosis but little acinar cell apoptosis (Bhatia 2004; Guzman and Rudnicki 2006). As shown in the present study, IHC results clearly showed that TRAF6 was mainly located in the pancreatic

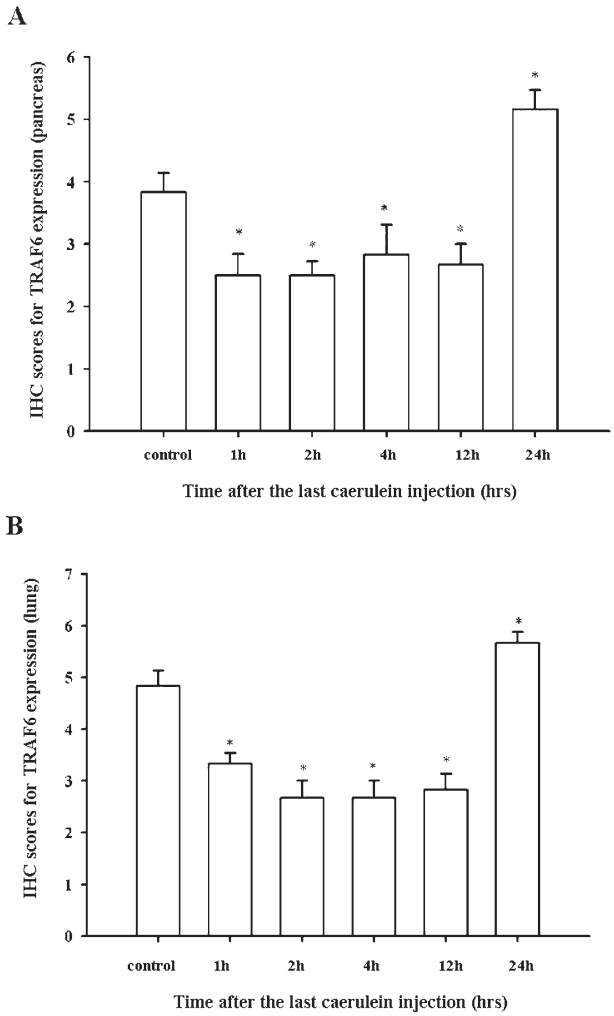

Fig. 4. Score of immunohistochemistry for TRAF6 in pancreas and lung.
TRAF6 protein expression was semiquantified. A, pancreas; B, lung. Results were expressed mean ± S.E.M (n = 6). *p < 0.05 vs control mice. Control represents the value at 1 h after saline injection.

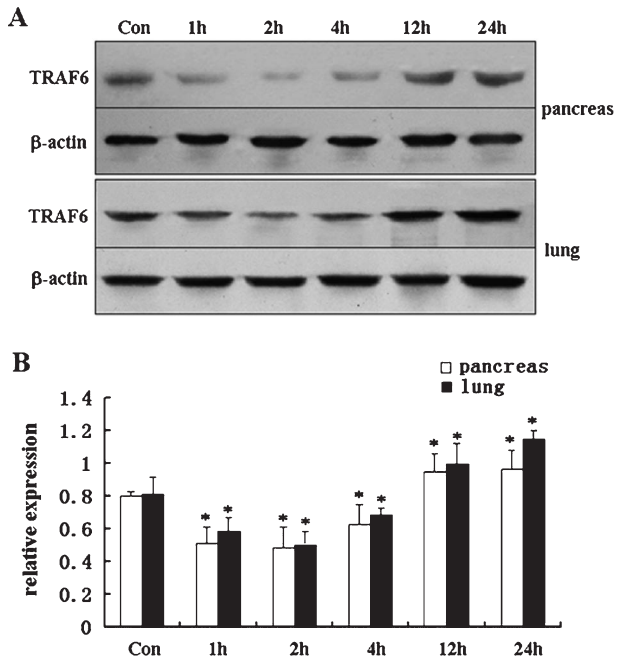

Fig. 5. Western blotting analysis of TRAF6 proteins levels of pancreas and lung.
A, representative Western blotting figure indicating the expression of TRAF6 protein. B, the semi-quantitative analysis of the data. Each column represents the average expression of TRAF6. *P < 0.05 vs the control ($n \geq 3$). Control represents the value at 1 h after saline injection. β-actin was used as loading control.

acinar cells. We therefore speculated that pancreatic acinar cells might be the main functional cells, when TRAF6 mediated pancreatic inflammation.

Acute lung injury is one of the critical complications of severe AP, but the molecular mechanism of acute lung injury during AP are still poorly understood (Gloor et al. 1998). Therefore, expression of TRAF6 in lung was also investigated in this study. The change of TRAF6 expression in lung was in accordance with those in pancreas, and TRAF6 was mainly located in bronchial epithelial cells. These data showed that TRAF6 was also important in the pathogenesis of pancreatitis-associated acute lung injury, and lung bronchial epithelial cell may be the main functional cell of TRAF6 associated lung inflammatory reaction.

The downstream products of the TLRs-TRAF6 signaling pathway, including pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 were determined by the state-of-the-art Luminex liquichip workstation. Luminex Liquichip assays enable the detection and quantitation of multiple cytokines simultaneously in one sample. Pro- and anti-inflammatory cytokines together formed a complicated network during AP, and disturbed balance between pro-inflammatory and anti-inflammatory cytokines determined clinical outcome of patients with AP (Altavilla et al. 2003; Simovic et al. 2007). Our result showed IL-6 and IL-10 were up-regulated within a short time-course, and the balancing mechanism between pro- and anti-inflammatory cytokines needs to be further investigated.

In conclusion, our study revealed that TRAF6 is involved in the regulation of pancreatic and lung inflammatory progress during AP for the first time. Although the present study clarified only a part of the mechanisms of action of TRAF6, these data might provide a possible strategy for preventing AP in clinical practice. Further investigations are required to elucidate the accurate mechanism, by which TRAF6 affects the inflammatory process during AP and acute lung injury.

Acknowledgments

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