

# Expression and roles of TIPE2 in autoimmune hepatitis

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**Abstract.** Tumor necrosis factor, alpha-induced protein 8-like 2 (TIPE2) is associated with the development of hepatic inflammatory diseases. However, to date, the possible role of TIPE2 in autoimmune hepatitis (AIH) has not been reported. The present study aimed to investigate the expression of TIPE2 in peripheral blood mononuclear cells (PBMCs) of mice with AIH. Furthermore, the liver function, pro-inflammatory cytokine production and hepatic histopathology were examined in TIPE2-deficient mice in order to evaluate whether TIPE2 is involved in the pathogenesis of AIH. A murine model of AIH was induced by treatment with concanavalin A (ConA). The expression of TIPE family members in the PBMCs was examined using reverse-transcription quantitative polymerase chain reaction analysis, while the protein expression of TIPE2 was additionally detected by western blot analysis. The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum was measured on an automated chemical analyzer to assess liver function. The serum levels of tumor necrosis factor- $\alpha$ , interleukin (IL)-6 and IL-12 were measured using commercial ELISA kits. Hematoxylin and eosin staining was performed to assess hepatic histopathology. The results showed that the expression of TIPE2 was significantly decreased in the mice with AIH. Following ConA-induced AIH, TIPE2-deficient mice had significantly increased serum ALT and AST levels, enhanced production of pro-inflammatory cytokines, as well as more severe hepatic inflammation compared with the wild-type mice. In conclusion, the present study demonstrated, for the first time, that TIPE2 is involved in the pathogenesis of AIH. TIPE2 prevents liver dysfunction and inhibits deleterious inflammatory immune responses after AIH and may therefore serve as a novel agent for the treatment of AIH.

## Introduction

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease characterized by interface hepatitis, increased levels of autoantibodies and immunoglobulins (Igs), as well as liver dysfunction (1-4). The disease has a universal distribution affecting all age groups and both genders, irrespective of the ethnicity of the affected individual (5-10). Although much progress has been made in the understanding of the pathogenesis of AIH in the past few years, the immunological mechanism of AIH remain to be elucidated.

The tumor necrosis factor-alpha (TNF- $\alpha$ )-induced protein-8 like-2 (TIPE2) is a newly identified negative regulator of immunity (11,12). It is highly expressed in hematopoietic cells (13,14), and negatively regulates inflammation through T cell receptor (TCR), Toll-like receptor (TLR), nuclear factor-kappa-B, c-Jun N-terminal kinase and p38 signaling pathways (11,13,15,16-18). The association between TIPE2 and liver diseases has been widely studied (19-24). It has been reported that TIPE2 has important roles in suppressing Hepatitis B virus (HBV) -induced hepatic inflammation. Patients with chronic hepatitis B had significantly reduced expression levels of TIPE2 in their peripheral blood mononuclear cells (PBMCs) compared with the healthy controls (22). Kong *et al* (23) reported that the TIPE2 mRNA expression was significantly downregulated in chronic hepatitis C patients, and HCV may promote chronic hepatitis by inhibiting TIPE2 expression. In patients with acute-on-chronic hepatitis B liver failure (ACHBLF) (24), the levels of TIPE2 mRNA correlated with serum total bilirubin and model for end-stage liver disease scores. TIPE2 mRNA levels were significantly upregulated in patients with ACHBLF compared with those in the patients with chronic infection or the healthy controls. Cytotoxic T cell-mediated killing of virus-infected hepatocytes is an important pathogenic process of hepatitis B. Recently, Zhang *et al* (21) reported that TIPE2 protein negatively regulates HBV-specific CD8 (+) T-cell functions in patients with hepatitis B. This suggests that TIPE2 may activate the destruction of hepatocytes by certain types of blood cell. However, to the best of our knowledge, the expression and the role of TIPE2 in AIH has not yet been reported.

In the present study, a murine model of concanavalin A (ConA)-induced AIH was established, and the expression of TIPE family members was assessed in the PBMCs of these mice. Furthermore, liver function, pro-inflammatory cyto-

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kine production and hepatic histopathology were assessed in TIPE2-deficient mice to evaluate whether TIPE2 is involved in the pathogenesis of AIH.

## Materials and methods

**Experimental animals.** A total of 72 wild-type (WT) C57BL/6 mice were purchased from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). The TIPE2-knockout (*TIPE2*<sup>-/-</sup>) mice with C57BL/6J background were generated as described previously (11). All mice used were male, 8-10 weeks old and weighed 22-25 g. The mice were maintained in individual cages under controlled conditions (22±2°C; 55% humidity; 12-h light/dark cycle), with free access to food and fresh water. All animal experiments were approved by the Ethics Committee of Hebei Province Chinese Medicine Hospital (Shijiazhuang, China) following the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 86-23, revised 1986). To induce AIH, the mice were injected with 2 mg/kg ConA (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) through the tail vein. Mice in the control group were injected with an identical volume of phosphate-buffered saline (PBS). Each group contained 6 mice.

**Collection of PBMCs.** Blood samples (~200 µl) were obtained via the tail vein at 6, 12 and 18 h after ConA-induced AIH. At 24 h, the mice were sacrificed via cervical dislocation and ~1 ml blood samples were harvested. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphocyte Separation Solution (Sigma-Aldrich; Merck Millipore) and stored at -80°C.

**Western blot analysis.** The PBMCs were lysed with radio-immunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) and the total protein concentration was determined using a Bradford Protein Concentration Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. A total of 30 µg protein was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk in Tris-buffered saline containing Tween 20 (TBST) at 4°C overnight, the membranes were incubated with the following primary antibodies at 4°C overnight: Rabbit polyclonal to TIPE2 (dilution, 1:500; cat. no., ab170258; Abcam, Cambridge, MA, USA), rabbit polyclonal to GAPDH (dilution, 1:2,000; cat. no. ab9485; Abcam). GAPDH antibody was used as an internal control. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution, 1:4,000; cat. no. ab6721; Abcam) at room temperature for 2 h. Detection of the signals was performed using an ECL Western Blotting kit (Pierce Biotechnology, Inc., Rockford, IL, USA) by exposure to X-ray films (Phenix Research Products, Candler, NC, USA) for 5 min. Protein was quantified using a ScanJet 4C Flatbed Scanner (Hewlett-Packard, Palo Alto, CA, USA) with NIH Image v1.52 software (rsb.info.nih.gov/nih-image/).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the PBMCs obtained at 12 h after ConA injection by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufac-

turer's instructions. Reverse transcription was performed with a First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). Real-time PCR was performed in an ABI 7300 Sequence Detection System with a SYBR-Green PCR kit (both Applied Biosystems; Thermo Fisher Scientific, Inc.). All primers used in this study were purchased from Sangon Biotech Co., Ltd., (Shanghai, China), and the sequences were as follows: TIPE, forward 5'-ACATGATGCGGACAGTGGTA-3 and reverse 5'-AGCTCCTGCCTCTAGGTTCC-3'; TIPE1, forward 5'-TGACCCCAAACACCCTATGT-3' and reverse 5'-TCTCCCAATTCTCCACAACC-3'; TIPE2, forward 5'-TGAAACTCAGGTCCGCTTCT-3' and reverse 5'-tCCTAGTGCTGCCTCCAACT-3'; TIPE3, forward 5'-GGCATTCTCTACCGGAACAA-3' and reverse 5'-GTCCTTGCACTCATGCAGAA-3'. For qPCR, 1 µl cDNA, 1 nM primers and 12.5 µl 2X SYBR Green were mixed to obtain a final volume of 25 µl. Thermal cycling was performed as follows: 40 cycles at 95°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec. Relative levels of gene expression were determined using the comparative C<sub>q</sub> method (25) with GAPDH as the control.

**Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).** The blood samples were centrifuged at 1,000 × g at 4°C for 15 min, and the serum was extracted and stored at -20°C. The levels of ALT and AST in the serum were measured using the ALT/AST Assay Reagent kit (Sichuan Maker Science Technology Co., Ltd, Chengdu, China) according to the manufacturer's instructions with colorimetric evaluation on an Automated Chemical Analyzer (7600; Hitachi, Tokyo, Japan).

**Cytokine measurement.** The levels of TNF-α, interleukin (IL)-6 and IL-12 in the serum were measured using commercial kits (R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's instructions.

**Hematoxylin and eosin (HE) staining.** The mice were sacrificed at 24 h after ConA-induced AIH using cervical dislocation method. Liver tissues were fixed in 4% paraformaldehyde (Beijing Dingguo Changsheng Biotechnology Co., Ltd, Beijing, China) for 24 h and embedded in paraffin. Tissue blocks were sectioned at 4 µm thickness and slices were baked at 60°C for 4 h. After removal of the paraffin by using xylene and a graded ethanol series, the sections were incubated with hematoxylin (Beyotime Institute of Biotechnology) for 5 min, followed by washing with water and staining with eosin (Beyotime Institute of Biotechnology) for 1 min. The slides were viewed under a microscope (E200; Nikon Corp., Tokyo, Japan).

**Statistical analyses.** Statistical analyses were performed using SPSS 19.0 software (International Business Machines, Armonk, NY, USA). Values are expressed as the mean ± standard deviation. Student's *t*-test was used to evaluate the statistical significance of differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Expression of TIPE genes in the PBMCs of mice with AIH.** To induce AIH, the WT mice were injected with ConA. 12 h later, the mRNA expression of TIPE genes in the PBMCs

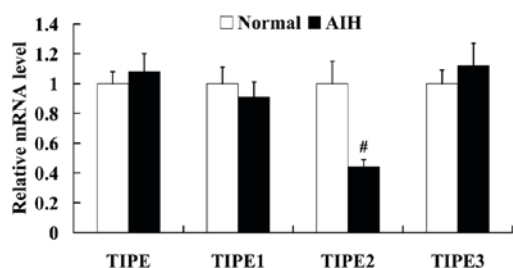


Figure 1. Expression of members of the TIPE family of genes in the peripheral blood mononuclear cells of mice with AIH at 12 h after concanavalin A injection. <sup>#</sup>P<0.05 vs. normal. TIPE, tumor necrosis factor- $\alpha$ -induced protein-8; AIH, autoimmune hepatitis.

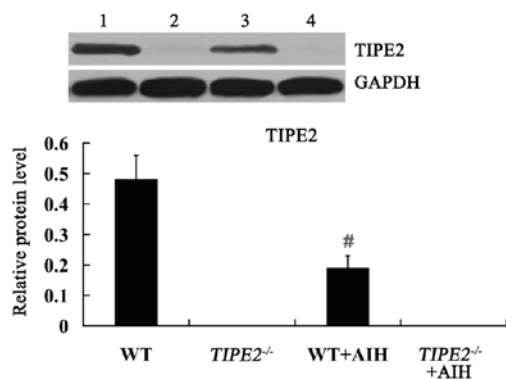


Figure 2. Expression of TIPE2 protein in the PBMCs of TIPE2-deficient mice with AIH at 12 h after concanavalin A injection. Lanes: 1, WT; 2, TIPE2<sup>-/-</sup>; 3, WT+AIH; 4, TIPE2<sup>-/-</sup>+AIH. <sup>#</sup>P<0.01 vs. WT. TIPE2, tumor necrosis factor- $\alpha$ -induced protein-8 like-2; AIH, autoimmune hepatitis; WT, wild-type.

was determined by RT-qPCR analysis. The results revealed that TIPE, TIPE1, TIPE2 and TIPE3 mRNA was detectable in the PBMCs of normal mice. The mRNA expression levels of TIPE2 were significantly decreased in the PBMCs of mice with AIH, while the mRNA expression levels of TIPE, TIPE1 and TIPE3 were not affected (Fig. 1).

**Expression of TIPE2 protein in the PBMCs of TIPE2-deficient mice with AIH.** The WT and TIPE2<sup>-/-</sup> mice were injected with ConA to induce AIH. 12 h later, the expression of TIPE2 protein in the PBMCs was examined by western blot analysis. As shown in Fig. 2, TIPE2 protein was not detectable in the PBMCs of TIPE2<sup>-/-</sup> mice, and that the relative protein levels of TIPE2 were significantly downregulated in the mice with AIH compared with the WT mice.

**Expression of ALT and AST in TIPE2-deficient mice with AIH.** The levels of ALT and AST in the serum were determined in order to evaluate liver function in TIPE2-deficient mice with AIH. As shown in Fig. 3, the levels of ALT and AST were significantly increased from 6 to 24 h post-ConA injection in the WT+AIH group compared with the WT group. Under normal conditions, there was no difference in ALT and AST levels between the WT and the TIPE2<sup>-/-</sup> mice. However, following ConA-induced AIH, the levels of ALT and AST in TIPE2<sup>-/-</sup> mice were higher compared with those in WT mice.

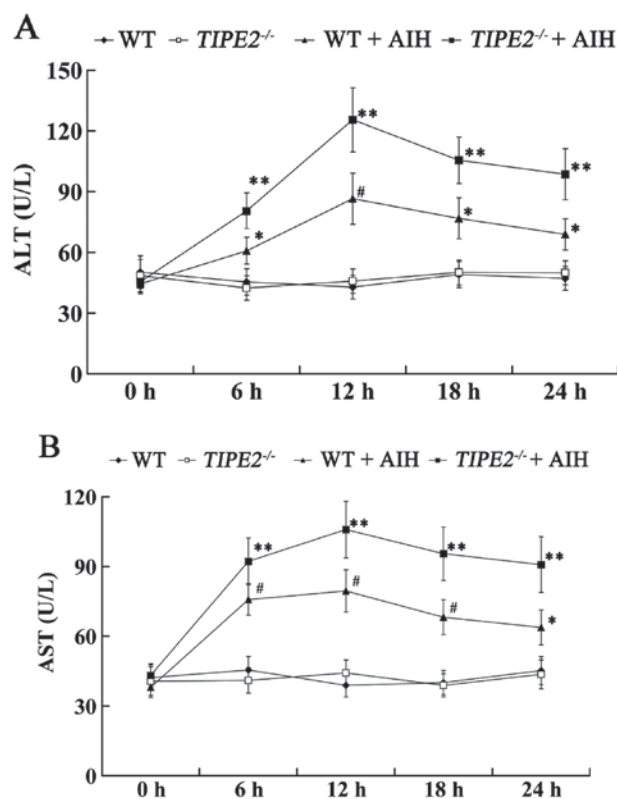


Figure 3. Expression of (A) ALT and (B) AST in the serum of TIPE2-deficient mice with AIH. <sup>#</sup>P<0.05 and <sup>#</sup>P<0.01 vs. WT; <sup>\*\*</sup>P<0.05 vs. WT+AIH. TIPE2, tumor necrosis factor- $\alpha$ -induced protein-8 like-2; AIH, autoimmune hepatitis; WT, wild-type; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

**Expression of pro-inflammatory cytokines in TIPE2-deficient mice with AIH.** The expression of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-6 and IL-12 in the serum were measured using ELISA. The expression of these pro-inflammatory cytokines was not significantly different between the WT and the TIPE2<sup>-/-</sup> mice prior to ConA-induced AIH. However, cytokine levels were significantly increased in WT mice following ConA-induced AIH from 6 to 24 h post-ConA injection. Furthermore, the levels of TNF- $\alpha$ , IL-6 and IL-12 were significantly increased in the TIPE2<sup>-/-</sup>+AIH group compared with those in the WT+AIH group (Fig. 4).

**Hepatic histopathology of TIPE2-deficient mice with AIH.** The HE staining of paraffin-embedded liver sections of the experimental mice is shown in Fig. 5. The liver structures of the WT and the TIPE2<sup>-/-</sup> mice appeared normal. Following ConA-induced AIH, hepatic injury was shown in the liver tissues. Mononuclear cell infiltration into the parenchyma, portal inflammation and hepatocellular necrosis were identified in the WT and the TIPE2<sup>-/-</sup> mice with AIH. The hepatic inflammation was more severe in the TIPE2<sup>-/-</sup>+AIH group than that in the WT+AIH group.

## Discussion

The family of TIPE proteins have been recently identified and consists of four members: TIPE, TIPE1, TIPE2 and TIPE3 (26), which have high degrees of sequence homology.

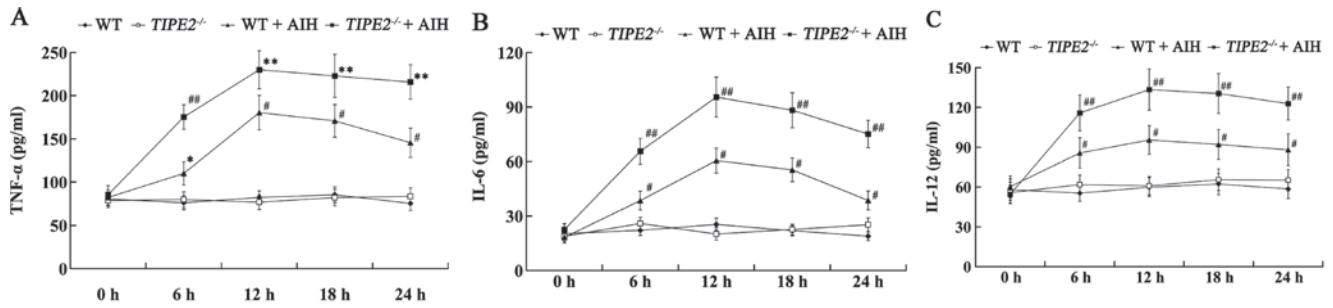


Figure 4. Expression of (A) TNF- $\alpha$ , (B) IL-6 and (C) IL-12 in the serum of TIPE2-deficient mice with AIH. \*P<0.05 and #P<0.01 vs. WT; \*\*P<0.05 and ##P<0.01 vs. WT+AIH. TIPE2, TNF- $\alpha$ -induced protein-8 like-2; AIH, autoimmune hepatitis; WT, wild-type; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL, interleukin.

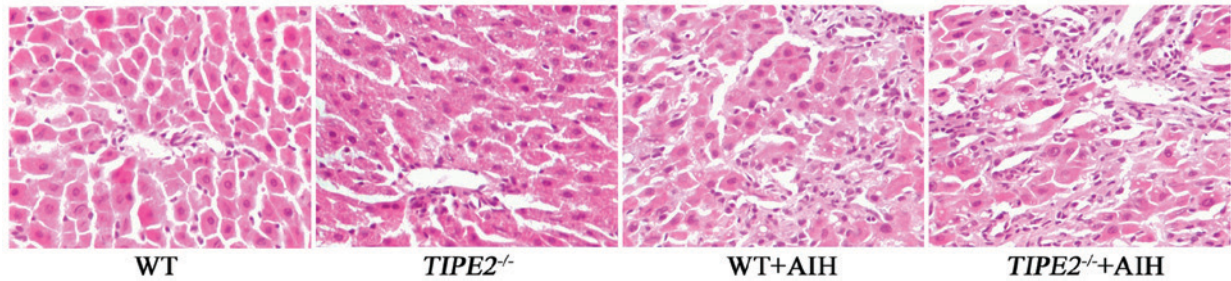


Figure 5. Hepatic histopathology of TIPE2-deficient mice with AIH. The cytoplasm was stained with eosin (red) and the nucleus was stained with hematoxylin (blue). (magnification, x400). TIPE2, tumor necrosis factor-alpha-induced protein-8 like-2; AIH, autoimmune hepatitis; WT, wild-type.

TIPE, the first identified member of this family, acts as a negative mediator of apoptosis and may have a role in tumor progression (12). The biological functions of TIPE1 and TIPE3 in physiological and pathological conditions have remained largely elusive. TIPE1 has been indicated to regulate cell death, and loss of TIPE1 may represent a novel prognostic indicator for hepatocellular carcinoma (27). TIPE3 is regarded as a transfer protein of lipid second messenger that promotes cancer (28). In the present study, the expression of TIPE, TIPE1 and TIPE3 in PMBCs was found to not be affected by ConA-induced AIH in experimental mice. These results indicated that TIPE, TIPE1 and TIPE3 may not be associated with AIH.

TIPE2 is the most widely studied protein of the TIPE family. It has essential roles in maintaining immune homeostasis (11,15). TIPE2 is down-regulated in patients with chronic inflammatory diseases, and TIPE2 deficiency in mice causes fetal inflammatory diseases (11). TIPE2 is also associated with the development of hepatic inflammatory diseases, including as HBV-induced hepatitis, HCV infection and ACHBLF (22-24). However, to the best of our knowledge, the role of TIPE2 in AIH has not been reported to date. In the present study, the expression of TIPE2 was found to be significantly decreased in mice with AIH. These data suggested that TIPE2 may have an important role in the pathogenesis of AIH.

ConA-induced hepatitis is a well-established model for the study of human AIH (29,30), as it mimics numerous aspects of this disease, including liver injury, pro-inflammatory cytokine overproduction (31). In the present study, to further investigate the role of TIPE2 in AIH, TIPE2-deficient mice were treated with ConA. Liver function, pro-inflammatory cytokine production and hepatic histopathology were then

examined, revealing that TIPE2-deficient mice had significantly increased serum ALT and AST levels, and enhanced the production of pro-inflammatory cytokines, as well as more severe hepatic inflammation compared with the WT mice, indicating that TIPE2 has a protective effect on liver function after AIH, and that TIPE2 may inhibit pro-inflammatory cytokine overproduction after AIH.

In conclusion, these findings of the present study demonstrated, for the first time, that TIPE2 is involved in the pathogenesis of AIH. The results suggested that TIPE2 is a regulator that prevents liver dysfunction and inhibits a deleterious inflammatory immune response after AIH. The present study suggested that TIPE2 may be a novel target for AIH treatment. Future studies should assess the role of TIPE2 in AIH in clinical studies, comparing the expression of TIPE2 between healthy controls and patients with AIH, and investigating the correlation between TIPE2 expression and clinical indices of patients with AIH.

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