Stable silencing of TIPE2 reduced the Poly I:C-induced apoptosis in THP-1 cells

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Abstract. The present study aimed to determine the underlying mechanism of toll-like receptor (TLR) agonist polyinosinic:polycytidylic acid (Poly I:C)-induced apoptosis in THP-1 cells following silencing the expression of tumor necrosis factor α-induced protein 8-like 2 (TIPE2). THP-1 cells were incubated with different concentrations of the TLR agonist. Following incubation, reverse transcription-quantitative polymerase chain reaction was performed to quantify the mRNA expression of TIPE2. Lentiviral technology was used to silence the expression of TIPE2. MTT assay was performed to assess cell proliferation, Annexin V/PI double staining was used to evaluate the apoptosis and western blotting was used to determine the expression levels of caspase-8 following TIPE2 silencing. The TLRs agonist Poly I:C increased the expression level of TIPE2. During the incubation, Poly I:C also inhibited the proliferation of THP-1 cells and induced apoptosis. Following silencing of TIPE2 in THP-1 cells, the Poly I:C-induced TIPE2 expression was significantly downregulated. Additionally, the Poly I:C-induced proliferation inhibition and apoptosis in THP-1 cells were significantly reduced following silencing of TIPE2. The findings of the western blot analysis indicated that the active form of caspase-8, p18, was downregulated following silencing of TIPE2. In conclusion, the expression of TIPE2 in THP-1 cells may be upregulated by Poly I:C, which may also inhibit cell proliferation and induce apoptosis. Following the downregulation of TIPE2 the aforementioned effect of Poly I:C treatment was reversed and may be associated with the reduced activity of caspase-8 that was observed in the TIPE2 silenced group.

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Introduction

The mechanism maintaining homeostasis in the immune system is very complex and remains to be fully elucidated. Homeostasis in the immune system may be disturbed by increased cell apoptosis or proliferation, which may lead to the development of fatal inflammatory disease. A previous study determined that a group of superfamily proteins are essential for the maintenance of the homeostasis in the immune system (1), particularly the protein with hexameric helix bundle structure termed death-effector domain (DED), similar to death domain and caspase activation and recruitment domain (CARD), which participate in apoptosis and other signaling pathways (2). Tumor necrosis factor α-induced protein 8-like 2 (TIPE2) has been previously identified to contribute to immune homeostasis (3). The primary mechanism is associated with the regulation of T cell receptors and toll-like receptor (TLR) signaling pathways (3,4). In addition to high expression in inflamed tissues, TIPE was also expressed in medullary tissue and a variety of tumor cells, indicating that it may have different functions (5). It has been previously reported that TIPE2 may regulate the expression of TLR (3). However, it remains to be determined whether the activation of upstream of TLR affects the expression of TIPE2 or the activity and proliferation of human monocytes. The present study was performed from January 2014 to December 2015 and used the THP-1 human monocyte cell line as the study subject, to investigate the effect of different TLR agonists on TIPE2 expression and the associated molecular mechanisms.

Materials and methods

Cell culture and TLR agonist treatments. THP-1 cells were purchased from Shanghai Cell Bank, Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China); newborn calf serum (NCS), Dulbecco's modified Eagle's medium (DMEM) and 0.25% trypsin were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA); human TLR1-9 agonists were purchased from InvivoGen, Inc.

(San Diego, CA, USA). Antibodies of human TIPE2 (cat. no. ab110389) and caspase-8 (cat. no. ab25901) were purchased from Abcam (Cambridge, MA, USA). Other reagents were domestic analytical grade. THP-1 cells were cultured in DMEM medium containing 10% NCS and 100 U/ml ampicillin, which was placed in an incubator at a temperature of 37˚C and 5% CO_2, and the medium was changed every 2-3 days. Cells in the logarithmic growth phase were selected for subsequent experiments. The cells were counted ($1x10^4$ cells per well), the TLR1-9 specific agonists were diluted with DMEM medium, resulting in the final concentration of 0.1 and 1 μg/ml. After 24 h incubation, cells were lysed using TRIzol (Thermo Fisher Scientific, Inc.). The RNA was extracted and the expression level of TIPE2 mRNA was detected as subsequently described.

Cytoinhibition experiments. The growth inhibition rate was analyzed using an MTT assay. THP-1 cells at a density of $1x10^4$/ml were seeded in 6-well or 12-well plates, placed in the incubator at 37˚C for 24 h. 10 mg/ml stock solution of polyinosinic:polycytidylic acid (Poly I:C) was diluted with culture medium and respectively added to at a final concentration of 1 or 0.1 μg/ml, and PBS was set as the control group. Cells were collected after 24 h treatment at 37˚C and 5 mg/ml MTT solution (Sangon Biotech Co., Ltd., Shanghai, China) was added and the mixture was incubated in 37˚C for 4 h. The supernatant was discarded and 150 μl DMSO was added to each well. Then the absorbance (OD) at 570 nm was detected using a microplate reader. The experiment was repeated for three times. The inhibition rate was calculated as follows: Inhibition rate = $(1 - OD_{ployI:C} / OD_{blank}) \times 100$. The detection of the growth inhibition rate following silencing TIPE2 expression of was similar to the aforementioned protocol. Briefly, 200 μl cells ($1x10^4$/ml) were seeded in 96-well plates, set as shTIPE2 silencing group (THP-1 cells after silencing the expression of TIPE2 screened using puromycin) and shScramble group (the empty vector screened using puromycin). After 24 h incubation with Poly I:C (1 μg/ml) at 37˚C, MTT was added to detect the absorbance (OD) at 570 nm.

Construction of lentiviral vector and the establishment of TIPE2-slienced cell lines. The synthetic oligonucleotides of shRNA (3 pairs of TIPE2 shRNA were synthesized by Sangon Biotech, Co., Ltd., (Shanghai, China) were designed and the specific sequences were as follows: shTIPE2-1 forward (F) 5'-CCGGGTGGCTCATCTCTTCATAGATCTCGAGATCTATGAAGAGATGAGCCACTTTTTG-3'; shTIPE2-1 reverse (R) 5'-AATTCAAAAAGTGGCTCATCTCTTCATAGATCTCGAGATCTATGAAGAGATGAGCCAC-3'; shTIPE2-2 F5'-CCGGTTCAATCTTCAGGCTTCATTCCTCGAGGAATGAAGCCTGAAGATTGAATTTTTG-3'; shTIPE2-2 5'-AATTCAAAAATTCAATCTTCAGGCTTCATTCCTCGAGGAATGAAGCCTGAAGATTGAA-3'; shTIPE2-3F 5'-CCGGGCCACGTGTTTGATCACTTCTCTCGAGAGAAGTGATCAAACACGTGGCTTTTTG-3'; shTIPE2-3 R'-AATTCAAAAAGCCACGTGTTTGATCACTTCTCTCGAGAGAAGTGATCAAACACGTGGC-3'. Double stranded DNA fragments were formed after annealing, and then linked to pLKO.1-TRC vector (includes GFP encoding sequence, BioVector NTCC Inc., Beijing, China) double digested by restriction endonucleases *Age*I and *Eco*RI. The 4 μl products was used to transform DH5α competent cells (BioVector NTCC Inc.) and incubated overnight at 37˚C. The 3 separated monoclonal colonies were inoculated into LB broth with ampicillin resistant, shaking at 37˚C with 8 x g overnight. Following the small extraction of plasmids by the SanPrep Column Plasmid Mini-Preps kit (Sangon Biotech Co., Ltd.), the combined plasmids were identified using a 1% agarose gel electrophoresis following digestion with *Eco*RI and *Nco*I enzymes. The identified plasmid was termed pLKO.1-TRC-TIPE2-shRNA1. The bacteria, which contained the confirmed plasmids were sent to Shanghai Shenggong Co., Ltd. (Shanghai, China) for sequencing.

For the packaged lentivirus, the 293T cells were divided the day prior to transfection. After combination of shTIPE2 or shScramble plasmid with the packaging plasmid pΔ8.91 and pMD2.G (BioVector NTCC Inc., the weight ratio of 10:10:1), Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) was mixed and incubated at room temperature for 20 min and then the total mixture was added to the 293T cells. Following incubation at 37˚C for 12 h, the medium was replaced with complete DMEM medium. After 48 h, the expression of green fluorescence was observed under fluorescence microscope. The supernatant was collected during 48-72 h and filtrated through a 0.45 μm filter. For the infection, THP-1 cells at logarithmic growth phase were selected and seeded into 6-wells ($1.5x10^5$ cells per well) for 12 h incubation at 37˚C. The supernatant of each well was discarded and 200 μl virus-containing liquid was added instead. Subsequently, the culture medium with polybrene (concentration of 4 μg/ml) was added for infection for 8 h at 37˚C, and then transferred the cells to the DMEM medium. After 48 h the culture was added to a medium with puromycin (concentration of 0.5 μg/ml) and the medium was replaced every 2-3 days. After screening for 2 months, normal medium was added and the successfully transfected cells were used for the subsequent experiments.

Detection of cell apoptosis. In order to determine the apoptotic rate, a Cell Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) was used for Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining. Following treatment with different concentrations of Poly I:C for 24 h, THP-1 cells were collected, washed with PBS and re-suspended with 300 μl binding buffer. Then 5 μl FITC-labeled Annexin V and 5 μl PI were added, and followed with addition of 200 μl binding buffer to the suspension. After incubation in dark at room temperature for 15 min, the mixture was filtered by 200-mesh nylon mesh and analyzed on BD Accuri C6 flow cytometer (BD Biosciences). The ratio of apoptotic cells and survival cells was calculated.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells from each treatment group were collected. The total RNA was extracted using TRIzol (Thermo Fisher Scientific, Inc.) and then the cDNA was synthesized using MMLV reverse transcriptase (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA from each group was used as the template for the subsequence PCR reaction. Primer sequences used were as follows: TIPE2, upstream 5'-GGAACATCCAAGGCAAGACTG-3', downstream 5'-AGCACCTCACTGCTTGTCTCATC-3'; GAPDH (internal

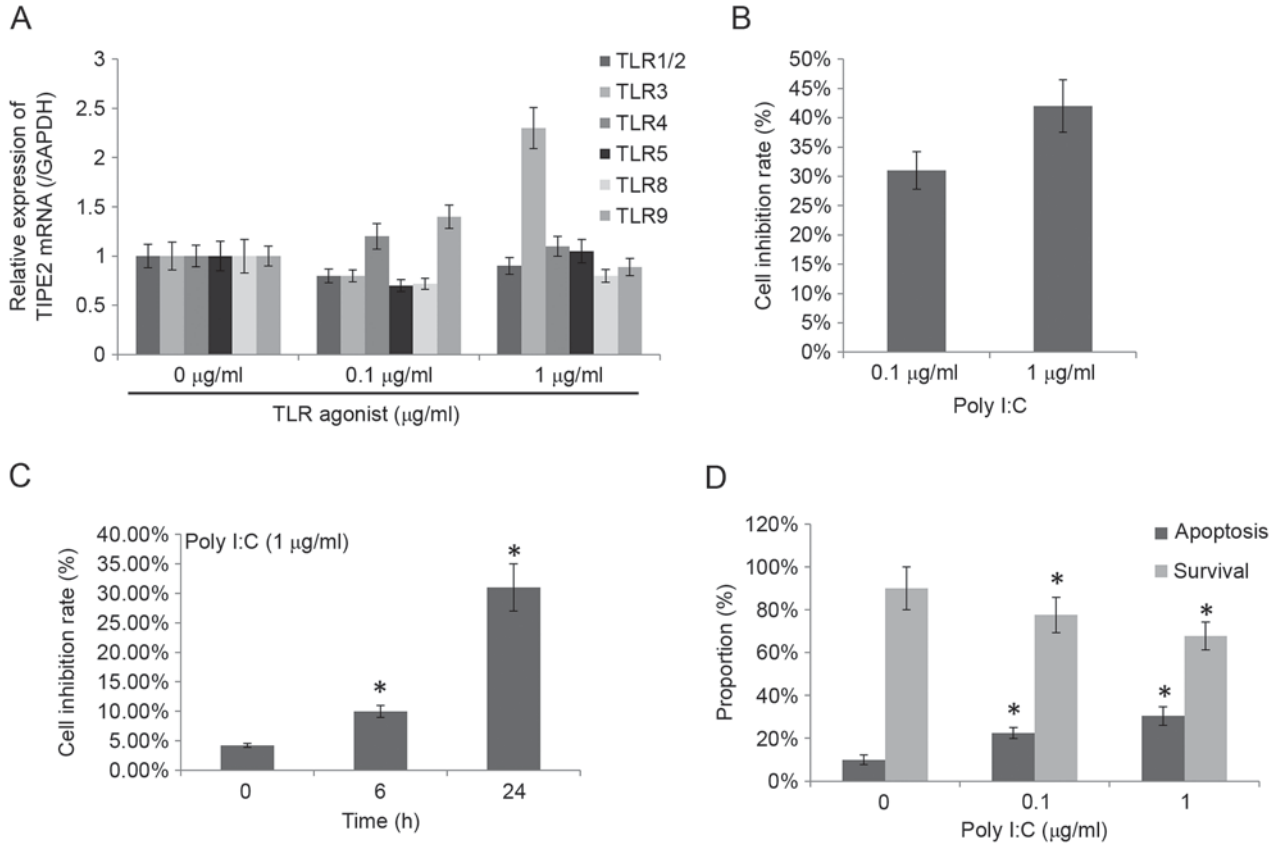

Figure 1. Effect of different TLR activators on the expression of TIPE2 and the influence of Poly I:C on the proliferation and apoptosis of THP-1 cells. (A) Following treatment of TLR1-9 for 24 h, the expression of TIPE2 in THP-1 cells was detected. (B) Cell proliferation inhibition when THP-1 cells treated with different concentrations of Poly I:C was detected using an MTT assay. (C) Cell proliferation inhibition of 1 μg/ml Poly I:C for different treatment times was detected by MTT assay. (D) Following Annexin V/PI double staining, the apoptosis rate following Poly I:C treatment was detected by flow cytometry. *P<0.05. TLR, toll-like receptor; TIPE2, tumor necrosis factor α-induced protein 8-like 2; Poly I:C, polyinosinic:polycytidylic acid.

control) upstream 5'-GTCGTATTGGGCGCCTGGTCACC-3', downstream 5'-CACACCCATGACGAACATGGGGGC-3'. The total reaction volume of 20 μl contained 2xSYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd., Dalian, China) 10 μl, upstream primer (10 μM) 0.5 μl, downstream primer (10 μM) 0.5 μl, cDNA 1 μl and ddH_2O 8 μl. The PCR thermocycling protocol was as follows: 95˚C pre-denaturation for 3 min; followed by 30 cycles of 95˚C for 10 sec, 55˚C for 30 sec, 72˚C for 30 sec. Each cDNA sample was measured in triplicate in the qPCR reaction, the mean of the quantification cycle (Cq) was used to calculate the ΔCq in each group (Cq target gene-Cq reference gene), and used the normal group as 1 for homogenization and the formula ($2^{-\Delta\Delta Cq}$) represented the relative expression level of the gene (6).

Immunoblotting. THP-1 cells were treated with Poly I:C for 24 h. Cells were washed once with cold PBS and lysed by RIPA buffer (Sangon Biotech Co., Ltd.). Following protein collection, the protein concentration was determined using a bicinchonic acid assay kit (Sangon Biotech Co., Ltd.). SDS-PAGE electrophoresis was performed with 20 μg protein/lane and then transferred onto PVDF membranes which were blocked with 5% non-fat milk. Primary antibodies, including TIPE2 (1:2,000, cat. no. ab110389), caspase-8 (1:2,000, cat. no. ab25901), GAPDH (1:4,000, cat. no. ab9485), β-actin (1:4,000, cat. no. ab8227) purchased from Abcam (Cambridge, UK) and p41/42 (1:2,000, cat. no. 9748) purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), were added. Following overnight incubation at 4˚C, the membrane was taken out and washed with TBST for three times, then incubated with HRP-labeled secondary antibody (1:10,000, cat. no. L3042-2) obtained from SAB Biotherapeutics, Inc. (Sioux Falls, SD, USA) at room temperature for 1 h. The membranes were washed with TBST three times and visualized using SuperSignal West Femto Trial kit (Thermo Fisher Scientific, Inc.). Relative expression of protein was scanned and calculated using LI-COR western blot imaging system (LI-COR Biosciences, Lincoln, NE, USA) and β-actin was used as internal control.

Statistical analysis. Significant difference analysis was performed by GraphPad Prism software version 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). The data was the mean of three independent experiments, and is presented as the mean ± standard deviation. When the data had a normal distribution, data from two groups was compared using independent samples Student's t-test and multiple groups comparison were performed by an analysis of variance (one-way ANOVA followed by Dunnett's post hoc test). If the data did not have a normal distribution, the data should be analyzed using the nonparametric Mann-Whitney test. P<0.05 was considered to indicate a statistically significant difference.

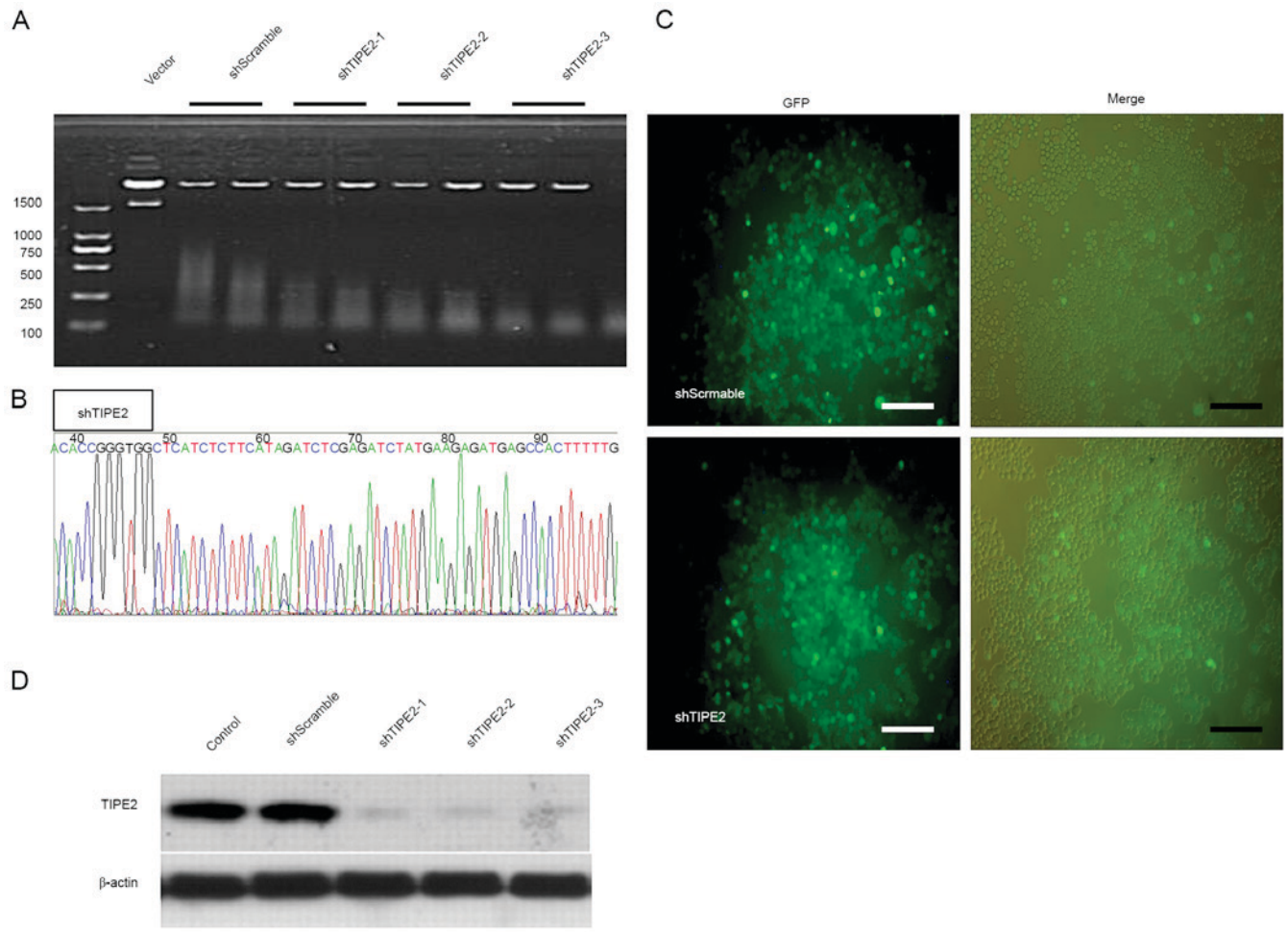

Figure 2. Construction of shTIPE2 lentiviral vector and the infection of THP-1 cells. (A) shRNA fragment was associated with pLko vector and then transformed to the competent cells. The plasmids were extracted and identified by enzyme digestion and 1% agarose electrophoresis. (B) The fragments of shRNA targeting TIPE2 were verified by Sanger sequencing. (C) Packaged shTIPE2 and shScramble virus infected THP-1 cells. (D) Transfected THP-1 cells were verified using protein expression analysis. shRNA, short hairpin RNA; TIPE2, tumor necrosis factor α-induced protein 8-like 2.

Results

Effect of different TLR agonists on TIPE2 expression. TIPE2 was affected by Poly I:C activation and the other specific activators revealed specific changes (Fig. 1A). However, the group treated with Poly I:C had the highest significant upregulation of TIPE2 expression (0.8±0.06 and 2.3±0.21 with the relative expression intensity of 0.1 and 1 μg/ml, respectively) and it was the main agonist for TLR3 specific agonist Poly I:C. A previous study revealed that Poly I:C may inhibit the proliferation of a variety of cells and induce apoptosis (7). The effect of different Poly I:C concentrations and different incubation times on the growth inhibition rate of THP-1 cells was detected using an MTT assay and the results are presented in Fig. 1B and C. Poly I:C significantly inhibited the THP-1 cells, with the growth inhibition rate reaching 42% at a concentration of 1 μg/ml Poly I:C and the rate increased with greater incubation time. Subsequently, using Annexin V/PI double staining to detect the effect of Poly I:C on THP-1 apoptosis, it was determined that the apoptotic cells gradually increased and the survival cells reduced significantly with greater Poly I:C concentration ($P<0.05$; Fig. 1D).

Construction of cell culture for silencing TIPE2 expression. Previous studies have revealed that TIPE2 may be involved in the activation of caspase-8 (3). In order to confirm that TLR3-specific inhibition of the THP-1 cells and the induced apoptosis by Poly I:C treatment may be associated with TIPE2 upregulation, the expression of TIPE2 in THP-1 cells was silenced using lentivirus technology (Fig. 2A). The constructed vector was transformed into competent cells DH5α. Following the extraction, the plasmids were identified by enzyme digestion, with the fragment sizes from the original plasmid digested to be 7,872, 2,155 and 190 bp, whereas the sizes from plasmids inserted with shRNA should be 7,872, 190, 303 and 42 bp. The plasmids with the appropriate digestion fragment sizes were sequenced for verification (Fig. 2B). The verified plasmids were selected for extraction and the endotoxin was removed for transfecting the packaging virus. As presented in Fig. 2C, the higher of the viral titer was, the better effect of infection was on THP-1 cells. The successful transfection of shTIPE2 was verified using western blotting (Fig. 2D), and it was revealed that the three types of shRNA designed successfully silenced TIPE2 expression.

Influence of silencing the expression of TIPE2 on Poly I:C-induced apoptosis. As presented in Fig. 3A, after using shRNA to silence the expression of TIPE2 in THP-1 cells, the TIPE2 expression in the shScramble group was higher

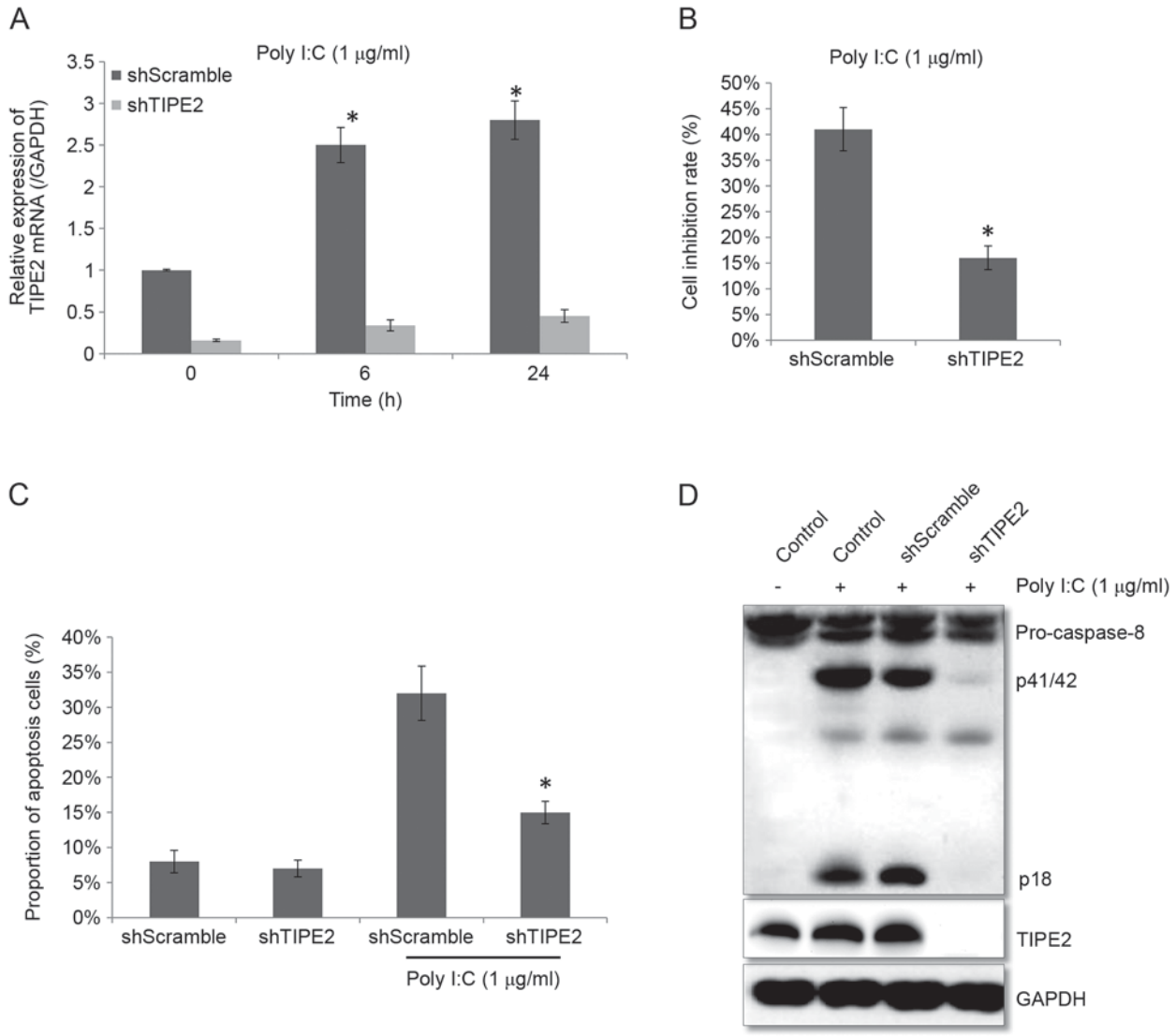

Figure 3. Response of silencing the expression of TIPE2 by Poly I:C-induced apoptosis. (A) The expression of TIPE2 in TIPE2-silenced cells after Poly I:C stimulation. (B) Silencing the expression of TIPE2 reduced the cell growth inhibition caused by Poly I:C treatment. (C) Poly I:C-induced apoptosis was also reduced after silencing of TIPE2 expression. (D) The active form of caspase-8 (p18) was detected by immunoblotting following silencing of TIPE2. *P<0.05 vs. shScramble.

when treated with Poly I:C, which may significantly increase the expression of TIPE2, whereas in shTIPE2 group, the upregulation of TIPE2 expression that was stimulated by Poly I:C treatment was significantly inhibited (P<0.05; Fig. 3A). Comparing the effect of Poly I:C treatment on the cell growth inhibition rate in the shScramble group with the shTIPE2 group revealed that the growth inhibition rate in the shScramble group was 41.2±4.3%, whereas the rate in the shTIPE2 group was significantly reduced at 16±2.3% (P<0.05; Fig. 3B). Apoptosis was subsequently detected by flow cytometry, which revealed that the percentage of apoptotic cells in the shTIPE2 group was 15.2±1.6% after Poly I:C treatment for 24 h, whereas in the shScramble group apoptotic cells were significantly greater at 32.2±3.9% (P<0.05; Fig 3C). Subsequently, expression of caspase-8 was determined to be reduced as the expression of p41/42 was decreased in shTIPE2 group. Additionally, western blot analysis revealed that the protein expression level of TIPE2 was significantly reduced in the shTIPE2 group, whereas that had no influence in shScramble group, suggesting that the silencing of the TIPE2 gene may reduce the apoptosis of THP-1 cells induced by Poly I:C.

Discussion

TLR is part of the innate immune-mediated transmembrane signaling receptor family, which has an important role in cell activation signal transduction, and links innate immunity with adaptive immunity. Previous studies revealed that TLR-conducted innate immunity had the same significance in the injury process of inflammatory cells induced by virus (8,9). TLR3 is an important member from TLR family, which is composed of extracellular leucine-rich repeats, transmembrane domain and cytoplasmic kinase domains (10). It can recognize specific double-stranded RNA (dsRNA) of pathogenic viruses. dsRNA viruses have pathogen associated molecular patterns, which may be identified by TLR3. During the replication period of a variety of viruses, large quantities of dsRNA were produced. Therefore, TLR3 may be used as an important anti-viral defense.

TLR3 contributes to the anti-viral process in the host and also has a close association with some autoimmune diseases. Previous studies determined that the RNA released by necrotic cells or mRNA produced from transcription *in vitro* may activate TLR3, indicating that the RNA released by necrotic cells may be used as the endogenous ligand to activate or regulate immune response (11). As most autoimmune diseases have not been identified to have a direct contact with the virus infection, endogenous ligands may have greater significance on the pathogenesis mechanism of TLR3 activation in autoimmune diseases (12).

TIPE2, as a new protein molecule, has partial sequences which are the similar to those of tumor necrosis factor-induced protein 8. TIPE2 is selectively expressed in lymphoid and myeloid-derived immune cells and it has a negative regulation on the innate immunity and cellular immunity. It may inhibit the activation of transcription factor activator protein-1 and nuclear factor-kB (3). Unlike the TIPE2 expressed primarily in mice lymphocytes, TIPE2 is expressed in various human tissues and cell types, including stem cells, neurons in the brain and brainstem, esophagus, cervical squamous cells, the junction of the bladder and urethra, colon, gastric epithelial cells and the appendix (13). It was identified to be expressed at high levels particularly terminal differentiated cells and its expression was downregulated in precursor cells, indicating that TIPE2 may be associated with the cell proliferation and differentiation (13). TIPE2 has been previously revealed to exhibit abnormal expression in various diseases, including hepatitis (14), liver cancer, stroke (15), kidney rejection (16), hepatitis C virus, asthma (17), myasthenia gravis (18), lupus erythematosus (19). The expression of TIPE2 in peripheral blood of the patients was reduced in a variety of autoimmune diseases, suggesting that immune cells were in a highly-activated state. It may aid in the reduction of the inhibition of autoimmune cell activity and the damage to normal tissues by specifically inducing the expression of TIPE2 in immune cells (20).

As an agonist of endogenous TLR3, Poly I:C may induce apoptosis in a variety of cell types, such as the epithelial cells of the bile duct and it may contribute to the inflammatory lesions of liver cirrhosis (21). Exogenous or endogenous dsRNA may induce various types of cell death via the TLR3 or IRF-3 pathway, including pancreatic β cell death (22), prostate cancer (23) and salivary gland epithelial cells (24). TLR3 is a pattern recognition receptor, and the apoptosis caused by TLR3 is primarily associated with caspase-8 (25). As an important chaperonin of caspase-8, TIPE2 has an essential role in its activity; therefore, it is considered that activation of caspase-8 and TIPE2 may be the underlying mechanism which triggers the apoptosis following Poly I:C-induced TLR3 activation. In addition, previous studies about tumors (26) have revealed that the expression of TIPE2 was reduced in some tumors and TIPE2 was the primary inhibitor of Ras, which inhibited the formation of Ras complex by inhibiting ral guanine nucleotide dissociation stimulator, ultimately leading to the inhibition of Ral and Akt. If the expression was upregulated, it may reduce the activation of Ras and in turn decrease cell proliferation and migration. Therefore, it is possible that that Poly I:C activates TLR3 and upregulates the expression of TIPE2, which may lead to further inhibition of Ras, thereby inhibiting cell proliferation. Poly I:C may influence the migration of cells and their phagocytic and antibiotic abilities. The cell proliferation and migration speed increased after following silencing of TIPE2 and it may inhibit the exocytosis, conversely after overexpression of TIPE2, it would suppress the hepatoma cell proliferation and the invasion *in vivo* and *in vitro* (27,28). Previous studies revealed that TIPE2 is associated with reducing Rac1 and F-actin levels and the activation of urokinase (29). The present study observed that THP-1 cell proliferation increased following silencing of TIPE2, which was consistent with previous studies (30).

Therefore, TIPE2 has an important role in autoimmune diseases and may also contribute to the development of tumors. Artificial upregulation of TIPE2 expression in tumor cells may inhibit the cancer cell proliferation and migration; however, it may also affect the activation of immune cells. Therefore, the potential negative impact on the normal immune system should be considered if the TIPE2 is used as a drug target in future cancer therapy.

Acknowledgements

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