

# TIM4/NLRP3 axis participates in the effects of M1 macrophages on inflammatory factor release, apoptosis and cell adhesion in thyroid follicular cells

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**Abstract.** Hashimoto's thyroiditis (HT) is an autoimmune thyroid disorder that predominantly affects women. The role of the T-cell immunoglobulin and mucin domain-containing 4 (TIM4)/ NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) signaling pathway in macrophages has previously been studied, but its effects on macrophage-mediated HT has not yet been reported. Therefore, the aim of the current study was to explore the regulatory role of TIM4/NLRP3 in the effects of M1 macrophages on the inflammation, apoptosis and cell adhesion of thyroid follicular cells. To induce M1 macrophage, 10 ng/ml of LPS and 20 ng/ml IFN- $\gamma$  were applied for the administration of THP-1 cells for 24 h. After induction, the mRNA expressions of M1 macrophage markers were assessed utilizing reverse transcription-quantitative (RT-q)PCR. Western blotting and immunofluorescence assay were adopted for the appraisal of inducible nitric oxide synthase. Additionally, the expression levels of TIM4 and NLRP3 before or after transfection were tested using RT-qPCR and western blotting. The release of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) were estimated using RT-qPCR and western blotting was adopted for the estimation of phosphorylated (p)-p65, p65, I- $\kappa$ B and p-I- $\kappa$ B. Furthermore, the apoptosis level as well as the accompanied proteins was appraised via TUNEL and western blotting. The mRNA and protein expressions of  $\alpha$ v $\beta$ 3 were evaluated employing RT-qPCR and western blotting. The results demonstrated

that TIM4 silencing decreased NLRP3 expression level in M1 macrophages. Moreover, TIM4 silencing in M1 macrophages reduced the expression levels of TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , as well as the phosphorylation levels of p65 and I $\kappa$ B in M1 macrophages co-cultured with Nthy-ori 3-1 cells, whereas NLRP3 overexpression significantly reversed these effects. Furthermore, NLRP3 overexpression reversed the decreased apoptotic rate and cell adhesion of Nthy-ori 3-1 cells induced by TIM4 silencing. In summary, the present study demonstrated that TIM4-silencing alleviated the inflammatory damage, apoptosis and cell adhesion of M1 macrophages co-cultured with Nthy-ori 3-1 cells through downregulation of NLRP3. Therefore, the regulation of M1 macrophages via the TIM4/NLRP3 axis may be a potential therapeutic approach for the treatment of patients with HT.

## Introduction

Hashimoto's thyroiditis (HT), an organ-specific autoimmune disease, is the most common thyroid disease that leads to hypothyroidism and has a high incidence rate of 5% in women in China (1). T helper 1 (Th1) cells were reported to be involved in the development of HT and HT is initially characterized by lymphocytic infiltration of the thyroid parenchyma, diffusely enlarged thyroid gland as well as elevated production of auto-antibodies (2). Therefore, it may be hypothesized that early therapeutic intervention could potentially prevent the development of this disease, and maintain the normal structure and function of the thyroid.

Macrophages are important innate immune cells in the body that widely exist in various tissues and organs, and serve a crucial role in the inflammatory response and tissue repair (3). Stimulated by cytokines, such as lipopolysaccharide (LPS) and IFN- $\gamma$ , macrophages polarize into M1 macrophages that serve a pro-inflammatory role via the secretion of inflammatory factors, such as TNF- $\alpha$ , IL-6 and IL-1 (4). M1 macrophages can also aggravate tissue inflammatory damage. For example, M1 macrophage-derived exosomes have been reported to aggravate experimental autoimmune neuritis via modulation of the Th1 response (5). Moreover, in the myocardial infarction microenvironment, M1 macrophage-derived exosomes can inhibit angiogenesis

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and exacerbate cardiac dysfunction (6). Estradiol has been shown to promote the activation of M1-like macrophages via cadherin-11, which can worsen the temporomandibular joint inflammation in rats (7). Furthermore, the co-culture of M1 macrophages with articular chondrocytes can exacerbate the apoptosis of articular chondrocytes (8). However, the role of M1 macrophages in HT is still unclear. It has previously been reported that macrophage infiltration occurred in an autoimmune thyroiditis model (9) and that macrophage migration inhibitory factor can recruit macrophages to inflammatory injury sites. Therefore, macrophage levels may be increased in thyroiditis tissues (10). Macrophage inflammatory protein-1 (MIP-1)  $\alpha$  and MIP-1 $\beta$  expression levels have been reported to be increased in HT tissues (11). Therefore, the regulation of macrophages could have an important role in HT research. T-cell immunoglobulin and mucin domain-containing 4 (TIM4) is expressed in macrophages and its overexpression can activate the release of inflammasomes in monocytes/macrophages (12). In liver Kupffer macrophages, TIM4 silencing can reduce C-C motif chemokine ligand 4-induced liver fibrosis, and in Kupffer macrophages overexpressing TIM4, reactive oxygen species are produced and mitochondrial autophagy is activated (13). NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) has also been reported to mediate cytokine secretion and pyroptosis, and is associated with autoimmune thyroiditis (14). Moreover, TIM4 was testified to regulate NLRP3 expression in macrophages (12,15). Despite the fact that the relationship between TIM4 and NLRP3 has been widely discussed, their function in HT mediated by macrophages is not clear. Therefore, the aim of the present study was to investigate whether TIM4 participated in the underlying mechanism of M1 macrophages in the inflammation, apoptosis and cell adhesion of thyroid follicular cells (TFCs), via the regulation of NLRP3.

## Materials and methods

**Co-culture model of M1 macrophages and Nthy-ori 3-1 cells.** The human monocyte leukemia THP-1 cell line was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. THP-1 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) containing 10% FBS (Beijing Solarbio Science & Technology Co., Ltd.), 100 U/l penicillin and 100 U/l streptomycin in an incubator at 37°C with 5% CO<sub>2</sub>. When cells reached 80% confluence, they were sub-cultured. The differentiation of THP-1 cells into macrophages was induced using 150 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich; Merck KGaA) for 24 h at room temperature in RPMI-1640 medium. M0 macrophages were induced according to a previous study (16). Subsequently, M1 macrophages were produced via the induction of macrophage polarization using IFN- $\gamma$  (20 ng/ml; R&D Systems China Co., Ltd.) and LPS (10 pg/ml; Sigma-Aldrich; Merck KGaA) for 24 h at room temperature. The immortalized human thyroid follicular Nthy-ori 3-1 cell line was purchased from the European Collection of Authenticated Cell Cultures (cat. no. 90011609) and was cultured in RPMI-1640 medium containing 10% FBS, 100 U/l penicillin and 100 U/l streptomycin in an incubator at 37°C with 5% CO<sub>2</sub>. For co-culture experiments,

the cell suspension was prepared using M1 macrophages and Nthy-ori 3-1 cells. M1 macrophages (1 $\times$ 10<sup>6</sup> cells/well) were seeded into the upper Transwell chamber (pore size, 0.4  $\mu$ m) which was pre-coated with Matrigel (BD Biosciences) and added with serum-free medium (Beijing Bitab Biotechnology Co., Ltd.) at room temperature for 24 h, whereas Nthy-ori 3-1 cells (1 $\times$ 10<sup>6</sup> cells/well) were cultured in the medium with 10% FBS in the lower Transwell chamber at room temperature for 24 h.

**Reverse transcription-quantitative PCR (RT-qPCR).** Nthy-ori 3-1 cells from each group were added into an Eppendorf tube and lysed using Trizol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RT reaction system was prepared using a PrimeScript<sup>™</sup> RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol and cDNA was produced. qPCR was performed using the 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The required thermocycling conditions were set as follows: Initial denaturation at 95°C for 8 min; denaturation at 95°C for 25 sec; annealing at 60°C for 30 sec; extension at 72°C for 30 sec; and final extension at 72°C for 10 min. The primer sequences for qPCR were as follows: TNF- $\alpha$ , 5'-ATGAGCACTGAAAGCATGATCCG-3' (forward) and 5'-AATGATCCCAAAGTAGACCTGCC-3' (reverse); IL-1 $\beta$ , 5'-GCACGATGCACCTGTACGAT-3' (forward) and 5'-CACCAAGCTTTTTTGCTGTGAGT-3' (reverse); IL-6, 5'-AGACTTGCCTGGTGA-3' (forward) and 5'-GCTCTGGCTTGTTC-3' (reverse); C-X-C motif chemokine ligand 10 (CXCL10), 5'-GTGGCATTCAAGGAGTACCTC-3' (forward) and 5'-GCCTTCGATTCTGGATTTCAGACA-3' (reverse); TIM4, 5'-ACAGGACAGATGGATGGAATACCC-3' (forward) and 5'-AGCCTTGTGTGTTCTGCG-3' (reverse); NLRP3, 5'-CTTCTCTGATGAGGCCCAAG-3' (forward) and 5'-GCAGCAAAGTGGAAAGGAAG-3' (reverse); integrin  $\alpha$ v, 5'-TGCCAGGGTCTTTCTACCTCT-3' (forward) and 5'-GGGTGCCTAGGAGCATTGT-3' (reverse); integrin  $\beta$ 3, 5'-ACCAGTAACCTGCGGATTGG-3' (forward) and 5'-TCCGTGACACACTCTGCTTC-3' (reverse); GAPDH, 5'-GAAGGTGAAGGTTCGAGTC-3' (forward) and 5'-GAAATGTTGATGGGATTTC-3' (reverse). The relative mRNA expression levels were quantified using the 2<sup>- $\Delta\Delta$ CT</sup> method (17) with GAPDH serving as an endogenous control.

**Western blotting.** Total protein was extracted from Nthy-ori 3-1 cells with RIPA lysis buffer (Beyotime Institute of Biotechnology) and then quantified with the use of bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). Subsequently, the proteins (50  $\mu$ g per lane) were separated via 8% SDS-PAGE. Proteins were then transferred onto PVDF membranes. The membranes were blocked using 5% skimmed milk for 2 h at room temperature, which was followed by incubation with the following primary antibodies at 4°C overnight: Inducible nitric oxide synthase (iNOS) (1:1,000; cat. no. ab178945), TIM4 (1:1,000; cat. no. ab47637), NLRP3 (1:1,000; cat. no. ab263899), phosphorylated (p)-p65 (1:1,000; cat. no. ab86299), p65 (1:1,000; cat. no. ab16502), I $\kappa$ B (1:1,000; cat. no. ab32518), p-I $\kappa$ B (1:1,000; cat. no. ab133462), Bax (1:1,000; cat. no. ab32503),

cleaved poly (ADP-ribose) polymerase (PARP) (1:1,000; cat. no. ab32064), PARP (1:1,000; cat. no. ab191217), Bcl-2 (1:1,000; cat. no. ab32124), GAPDH (1:2,500; cat. no. ab9485) (all from Abcam) and  $\alpha\beta3$  (1:50; cat. no. #SC7312; Santa Cruz Biotechnology, Inc.) was detected as an entire protein band under the conditions used; Molecular Weight of Integrin  $\alpha\beta3$ : 125 kDa). Following the primary antibody incubation, HRP-labeled goat anti-rabbit (1:2,000; cat. no. ab6721; Abcam) or goat anti-mouse (1:2,000; cat. no. ab6789; Abcam) secondary antibodies were added and incubated with the membrane at room temperature for 30 min. GAPDH was used as the internal control. Finally, the protein band were visualized with ECL Detection Reagent (Shanghai Yeasen Biotechnology Co., Ltd.) and ImageJ software (version 7.6.5; National Institutes of Health) was used for the densitometric analysis of the protein expression levels.

**Immunofluorescence assay.** M1 macrophages ( $1 \times 10^6$  cells/well) that were inoculated into six-well plates were fixed using 4% paraformaldehyde for 15 min at room temperature. The cells were then permeated using 0.5% Triton X-100 for 30 min at room temperature. Subsequently, cells were rinsed with PBS and were then incubated with 5% goat serum (Beyotime Institute of Biotechnology) at room temperature for 30 min. The primary antibody, anti-iNOS (1:50; cat. no. ab3523; Abcam), was added to the cells at 4°C overnight. Following the primary antibody incubation, a goat anti-rabbit IgG H&L (Alexa Fluor® 488) secondary antibody (1:200; cat. no. ab150077; Abcam) was added for 1 h at 37°C. The nuclei were stained using a DAPI solution for 5 min at room temperature. Finally, the cells were mounted using an anti-fluorescence quenching agent and the images were observed using a fluorescence microscope.

**Transfection.** Short hairpin RNA (shRNA) targeting TIM4 (shRNA-TIM4-1, 5'-GTTCAACGATGTAAAGATA-3'; shRNA-TIM4-2, 5'-GGTACTTTAGAGACCACAA-3'), the corresponding empty vector [shRNA-negative control (NC), 5'-CCGGCAACAAGATGAAGAGCACCAACTC-3'], an NLRP3 overexpression plasmid (Ov-NLRP3) or its empty vector (Ov-NC) were obtained from Shanghai GenePharma Co., Ltd. Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) was used to transfect the aforementioned vectors into M1 macrophages at a concentration of 50 ng/ml at 37°C for 24 h. After 48 h, the cells were adopted for follow-up experiments.

**TUNEL staining.** Nthy-ori 3-1 cells ( $1 \times 10^6$  cells/well) that were inoculated into six-well plates were fixed using 4% paraformaldehyde at 4°C for 25 min. Subsequently, cells were incubated with proteinase K at room temperature for 5 min. The apoptotic cells were stained using TUNEL solution (Elabscience Biotechnology, Inc.) at 37°C for 1 h according to the manufacturer's protocol. Subsequently, 1  $\mu$ g/ml DAPI was applied for the staining of cell nuclei for 30 min at room temperature in the dark. A fluorescent microscope was adopted for the observation of positive cells.

**Cell adhesion assay.** Fibronectin (Shanghai Yeasen Biotechnology Co., Ltd.) was added to each well of a 96-well

plate and incubated at 4°C overnight. PBS was then used to wash the plate and 1% BSA (Beyotime Institute of Biotechnology) was added to each well for incubation for 1 h at room temperature. Subsequently,  $2 \times 10^4$  Nthy-ori 3-1 cells were added to each well for incubation for 30 min at 37°C. Adherent cells were fixed using 3% paraformaldehyde for 10 min at room temperature and stained with 0.5% crystal violet for 10 min at room temperature. The number of cells was analyzed using a spectrophotometer at 540 nm. A total of five randomly chosen fields were counted for each group.

**Statistical analysis.** The experimental data are presented as the mean  $\pm$  SD. SPSS v21.0 (IBM Corp.) was used for statistical analysis. The comparison between the two groups was performed using the unpaired Student's t-test. One-way ANOVA was performed for statistical comparisons among more than two groups followed by Tukey's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**M1 macrophages express decreased NLRP3 levels following TIM4 silencing.** M0 macrophages were polarized into M1 macrophages using 10 pg/ml LPS and 20 ng/ml IFN- $\gamma$ . The mRNA expression levels of M1 macrophage markers, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and CXCL10 were assessed via RT-qPCR. Their levels were significantly increased compared with in the M0 macrophage control group (Fig. 1A). Furthermore, western blotting and immunofluorescence staining determined that iNOS protein expression levels were markedly increased in the M1 macrophage group (Fig. 1B and C). Subsequently, the results demonstrated that the expression levels of TIM4 and NLRP3 were significantly increased in M1 macrophages compared with in the control group (Fig. 2A and B). To further investigate the role of TIM4, the effects of TIM4 silencing were assessed in M1 macrophages using RT-qPCR and western blotting. Compared with in the control group, the mRNA and protein expression levels of TIM4 were markedly reduced in the TIM4 knockdown groups (Fig. 2C and D). Moreover, it was observed that shRNA-TIM4-1 resulted in a more efficient TIM4 knockdown; therefore, shRNA-TIM4-1 was selected for use in the subsequent experiments. NLRP3 mRNA and protein expression levels were also assessed using RT-qPCR and western blotting. The results demonstrated that NLRP3 expression levels were significantly decreased following TIM4 silencing (Fig. 2E and F).

**Co-culture of M1 macrophages and Nthy-ori 3-1 cells leads to increased expression levels of inflammatory factors and apoptosis.** To assess whether inflammatory factor expression levels in Nthy-ori 3-1 cells following co-culture with M1 macrophages were related to TIM4/NLRP3, the mRNA expression levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6, and the protein expression levels of p-p65, p65, I $\kappa$ B and p-I $\kappa$ B were determined via RT-qPCR and western blotting, respectively. Furthermore, the apoptotic rate was determined via TUNEL staining and western blotting. M1 macrophages were transfected with Ov-NLRP3. The results demonstrated that the Ov-NLRP3 cell group exhibited increased expression

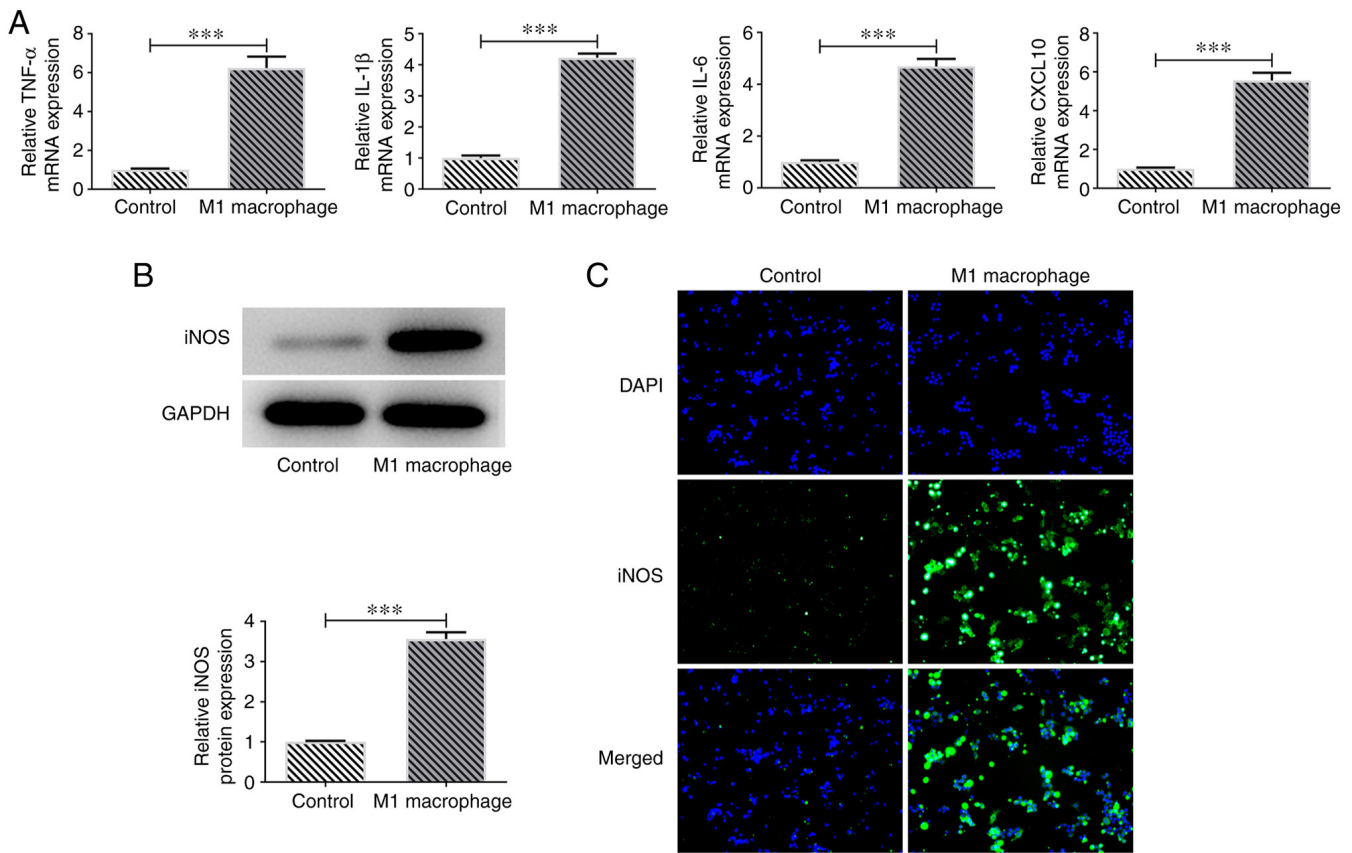


Figure 1. Polarization of M1 macrophages. (A) Detection of M1 markers, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and CXCL10, in M1 macrophages by reverse transcription-quantitative PCR. (B) Detection of iNOS expression levels by western blotting. (C) Immunofluorescence staining for iNOS. Data are shown as the mean  $\pm$  standard deviation of three independent experiments. \*\*\* $P$ <0.001. CXCL10, C-X-C motif chemokine ligand 10; iNOS, inducible nitric oxide synthase.

levels of NLRP3 compared with the M1-m+Ov-NC group (Fig. 3A and B). Moreover, the mRNA expression levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in Nthy-ori 3-1+M1-m group were significantly increased compared with those in the Nthy-ori 3-1 group (Fig. 3C). Compared with the Nthy-ori 3-1 + M1-m + shRNA-NC group, the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were greatly declined by TIM4 knockdown, which were then elevated by NLRP3 overexpression. The protein expression levels of p/t-p65 and p/t-I- $\kappa$ B in Nthy-ori 3-1 + M1-m group were significantly increased compared with those in Nthy-ori 3-1 group (Fig. 3D). The apoptotic rate was also significantly increased in Nthy-ori 3-1 + M1-m group when compared with the Nthy-ori 3-1 group (Fig. 4A and B). Additionally, the reduced apoptosis in thy-ori 3-1 + M1-m + shRNA-TIM4 group due to TIM4 depletion were promoted by Ov-NLRP3 in comparison with the thy-ori 3-1 + M1-m + shRNA-TIM4 + Ov-NC group. The results demonstrated that the protein expression levels of Bax and cleaved PARP were increased, and Bcl-2 protein expression levels were decreased, following the co-culture of M1 macrophages with Nthy-ori 3-1 cells compared with the Nthy-ori 3-1 cells group (Fig. 4C).

A previous study reported that IFN- $\alpha$  can inhibit the expression of integrin  $\alpha$ v $\beta$ 3 in Nthy-ori 3-1 cells and reduce their adhesion (18). To investigate whether the modulation of TIM4/NLRP3 in M1 macrophages regulated the adhesion of Nthy-ori 3-1 cells, M1 macrophages were transfected with plasmids inducing TIM4 silencing

(shRNA-TIM4) or NLRP3 overexpression (Ov-NLRP3). The number of Nthy-ori 3-1 cells after co-culture of M1 macrophages and Nthy-ori 3-1 cells was found to be higher compared with in the Nthy-ori 3-1 group (Fig. 5A). Furthermore, the number of attachment macrophages in thy-ori 3-1 + M1-m + shRNA-NC group were reduced by TIM4 deficiency, which was then increased by NLRP3 overexpression (Fig. 5B). Moreover, RT-qPCR and western blotting demonstrated that TIM4 silencing markedly elevated the mRNA and protein expression levels of  $\alpha$ v $\beta$ 3 in comparison with the thy-ori 3-1 + M1-m + shRNA-NC group, while NLRP3 overexpression exhibited opposite impacts on  $\alpha$ v $\beta$ 3, evidenced by the reduced expression of  $\alpha$ v $\beta$ 3 in thy-ori 3-1 + M1-m + shRNA-TIM4 + Ov-NC group (Fig. 5C and D).

## Discussion

HT is an organ-specific autoimmune disease caused by genetic, environmental and immune tolerance factors (19). Abnormal changes in the thyroid or immune system can lead to the disruption of immune tolerance, which can trigger an autoimmune response (20). However, at present, the exact pathogenesis of HT has not been fully elucidated. The present study demonstrated that M1 macrophages differentiated and polarized from THP-1 monocytes, and modulated the inflammatory and apoptotic response of Nthy-ori 3-1 cells via the TIM4/NLRP3 axis. An *in vitro* M1 macrophage and TFC co-culture model



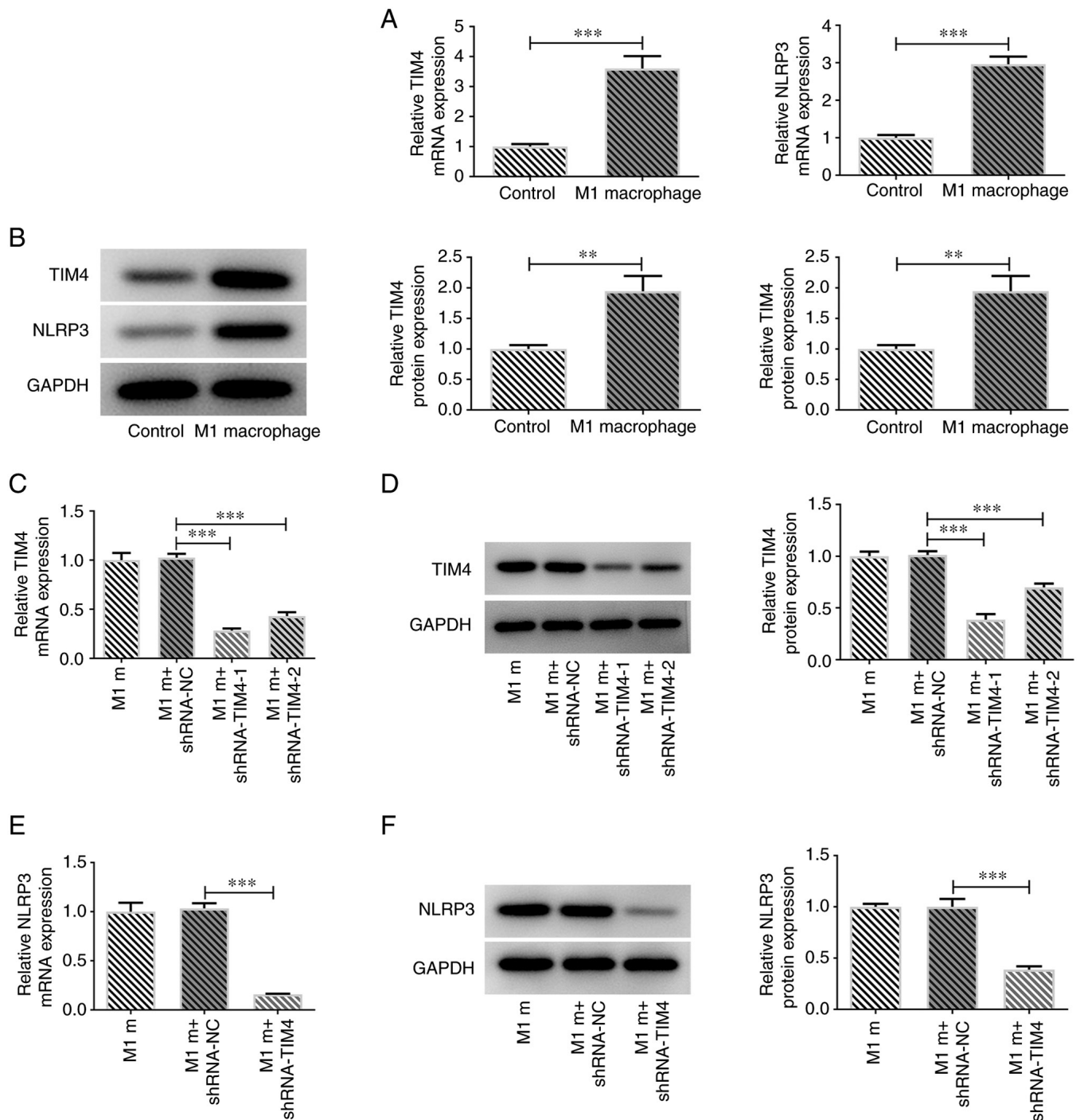


Figure 2. TIM4 silencing in M1 macrophages reduces NLRP3 levels. The expression levels of TIM4 and NLRP3 in M1 macrophages were evaluated by (A) RT-qPCR and (B) western blotting. TIM4 expression was analyzed after transfection with shRNA-TIM4-1/2 by (C) RT-qPCR and (D) western blotting. NLRP3 expression was analyzed after transfection with shRNA-TIM4 by (E) RT-qPCR and (F) western blotting. Data are shown as the mean  $\pm$  standard deviation of three independent experiments. \*\*P<0.01, \*\*\*P<0.001. TIM4, T-cell immunoglobulin and mucin domain-containing 4; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA; M1-m, M1 macrophage; NC, negative control.

was used to simulate the effects of macrophages observed in clinical or *in vivo* studies.

A previous study reported that there was an increased number of M1 macrophages in the mice model of thyroiditis relative to the control group and that a decrease in M1 macrophage polarization could produce a therapeutic effect (21). In the present study, the results demonstrated that M1 macrophages exhibited increased TIM4 and NLRP3 expression levels, which significantly affected

the inflammation and apoptosis of Nthy-ori 3-1 cells. A previous study demonstrated, via the analysis of microarray expression profiles of HT and normal thyroid samples, that genes associated with inflammation and apoptosis were dysregulated (22). The present study indicated that this dysregulation was potentially related to the modulation of M1 macrophages via the TIM4/NLRP3 axis. The present study also explored the effect of M1 macrophages on the adhesion of Nthy-ori 3-1 cells. TIM4 silencing resulted

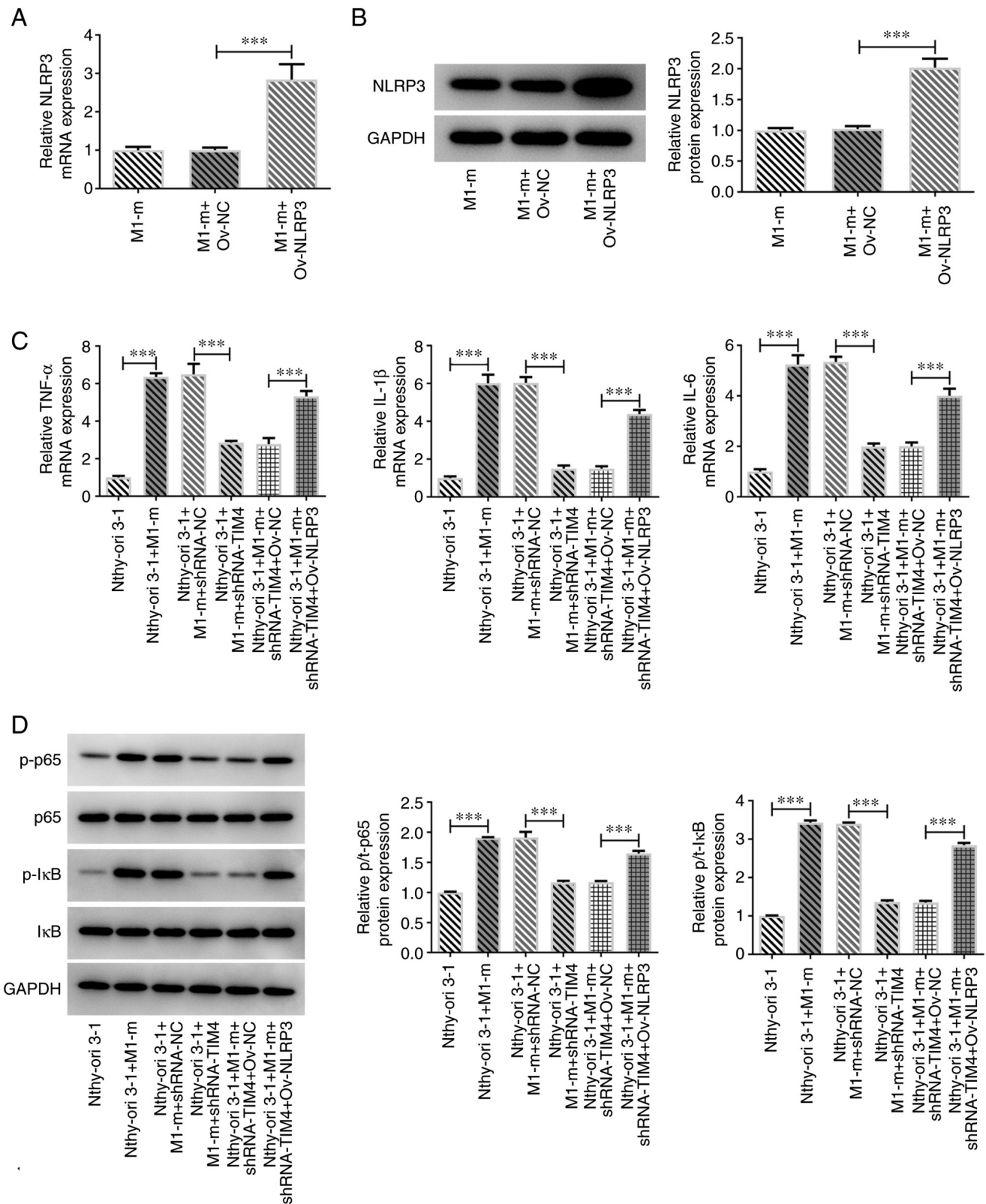


Figure 3. Overexpression of NLRP3 in M1 macrophages affects the expression of inflammatory factors in Nthy-ori 3-1 cells. NLRP3 expression in M1 macrophages after transfection with Ov-NLRP3 was determined by (A) RT-qPCR and (B) western blotting. (C) RT-qPCR analysis showing expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in Nthy-ori 3-1 cells after transfection. (D) Expression of p-p65, p65, I $\kappa$ B and p-I $\kappa$ B, detected by western blotting. Data are shown as the mean  $\pm$  standard deviation of three independent experiments. \*\*\* $P$ <0.001. TIM4, T-cell immunoglobulin and mucin domain-containing 4; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; Ov, overexpression plasmid; RT-qPCR, reverse transcription-quantitative PCR; M1-m, M1 macrophage; NC, negative control; shRNA, short hairpin RNA; p, phosphorylated; t, total.

in a decreased cell number, whereas NLRP3 overexpression markedly reduced this effect. Furthermore, it was

demonstrated that TIM4 silencing in M1 macrophages significantly increased the protein expression levels of

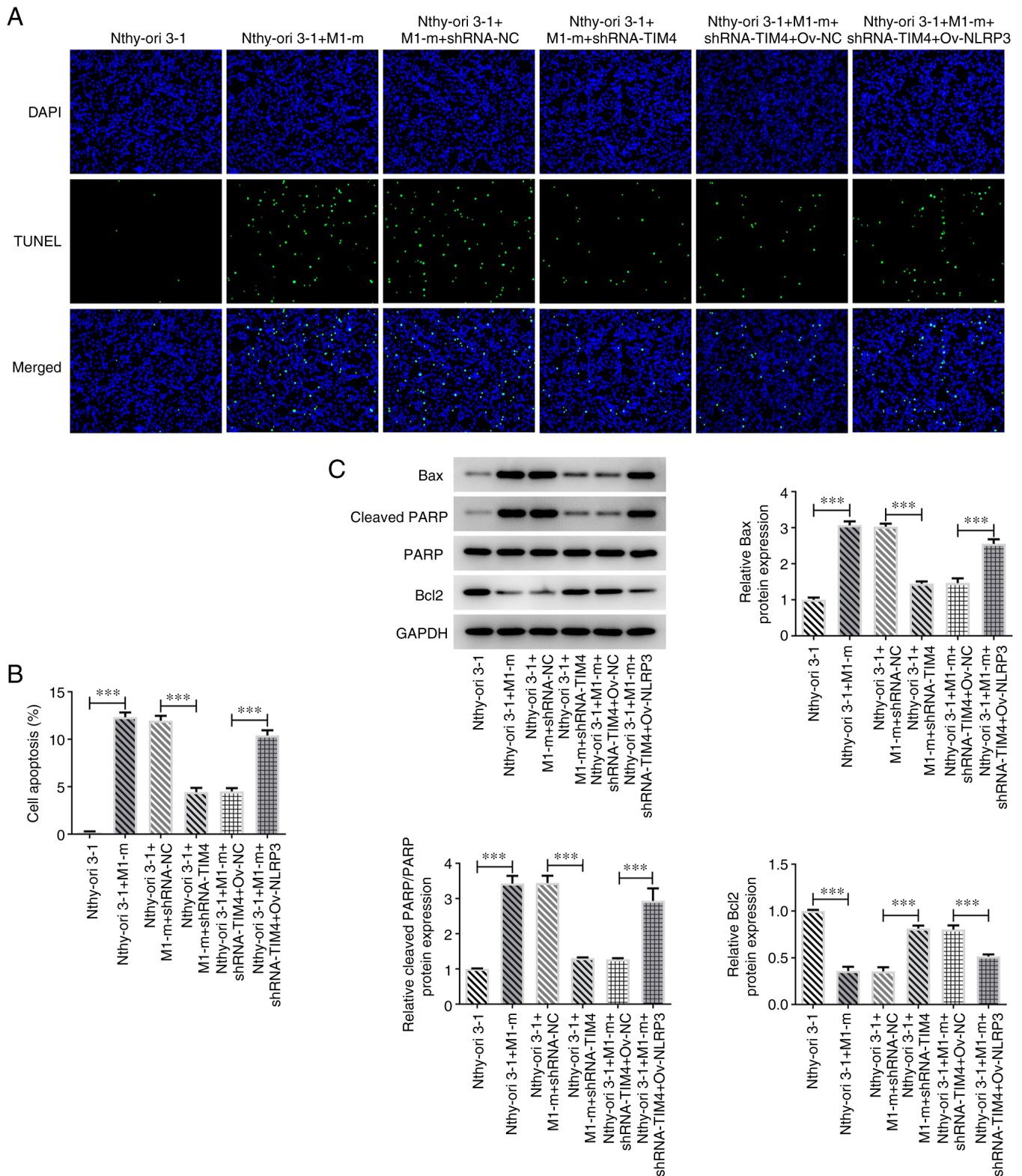


Figure 4. Overexpression of NLRP3 in M1 macrophages affects apoptotic levels in Nthy-ori 3-1 cells. (A) TUNEL staining and (B) quantification of apoptotic levels in Nthy-ori 3-1 cells transfected with shRNA-TIM4, with or without Ov-NLRP3. (C) Expression levels of apoptosis-related proteins were detected via western blot analysis. Data are shown as the mean  $\pm$  standard deviation of three independent experiments. \*\*\* $P$ <0.001. NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; shRNA, short hairpin RNA; TIM4, T-cell immunoglobulin and mucin domain-containing 4; Ov, overexpression plasmid; M1-m, M1 macrophage; NC, negative control; PARP, poly (ADP-ribose) polymerase.

integrin  $\alpha\text{v}\beta 3$  in Nthy-ori 3-1 cells. However, its effects were reversed by NLRP3 overexpression, which suggested that factors secreted by M1 macrophages potentially affected the adhesion of Nthy-ori 3-1 cells. In addition, the

present study demonstrated that TIM4 silencing resulted in decreased expression of NLRP3 in M1 macrophages, but the mechanism explaining the relationship between the two molecules is not clear and should be explored in the future.



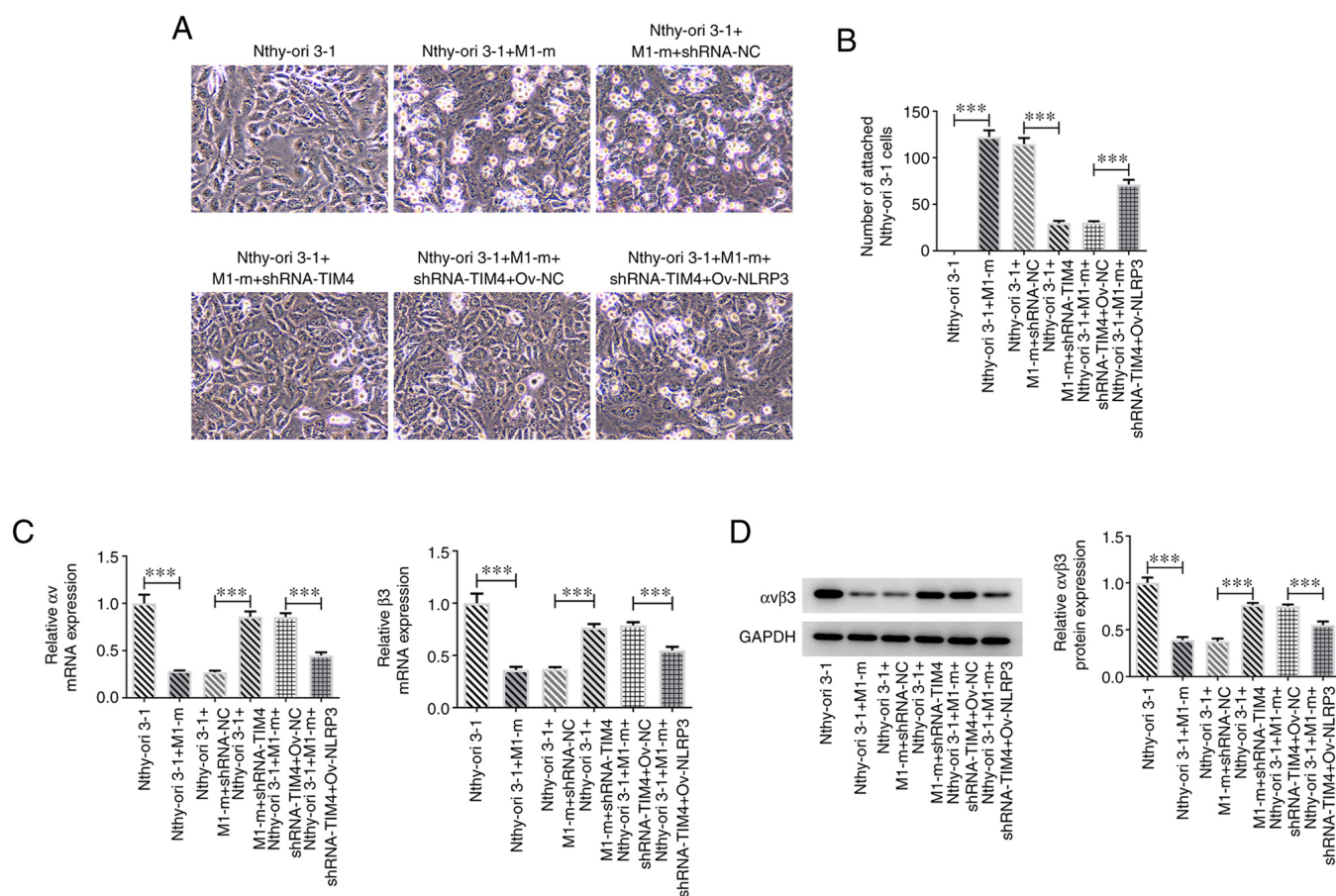


Figure 5. Overexpression of NLRP3 in M1 macrophages affects Nthy-ori 3-1 cell adhesion. (A and B) Adhesive capacity of Nthy-ori 3-1 cells co-cultured with M1 macrophages transfected with shRNA-TIM4, with or without Ov-NLRP3. The expression of integrin  $\alpha 5$  in M1 macrophages transfected with shRNA-TIM4, with or without Ov-NLRP3, was determined by (C) reverse transcription-quantitative PCR and (D) western blotting. Data are shown as the mean  $\pm$  standard deviation of three independent experiments. \*\*\* $P < 0.001$ . NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; shRNA, short hairpin RNA; TIM4, T-cell immunoglobulin and mucin domain-containing 4; Ov, overexpression plasmid; M1-m, M1 macrophage; NC, negative control.

In summary, to the best of our knowledge, the model used in the present study is novel, as THP-1 cells were used to produce M1 macrophages, which were then co-cultured with Nthy-ori 3-1 cells. Based on the results in the present study, it could be speculated that soluble factors secreted by M1 macrophages could activate inflammatory and apoptotic signaling pathways, and might be involved in the adhesion of Nthy-ori 3-1 cells to potentially induce cell injury. The model produced in the present study has provided a novel approach to study the modulation of the underlying signaling pathways in the effect of macrophages on Nthy-ori 3-1 cells in co-culture. Therefore, the regulation of M1 macrophages via targeting of the TIM4/NLRP3 axis may be a potential therapeutic approach for the treatment of HT.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

YC and YS designed the study, performed the experiments, and drafted and revised the manuscript. XJ, XL, LC and YQ analyzed the data and searched the literature. YC and YS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.



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