

# Effect of TNFAIP8 on the immune function of Th17 cells via p53/ p21/ MDM2 pathway after acute insult

**Xiaobin Cheng**

Hubei Provincial Hospital of Traditional Chinese Medicine

**Min Wang**

Hubei Provincial Hospital of Traditional Chinese Medicine

**Jing Li**

Hubei Provincial Hospital of Traditional Chinese Medicine

**Gang Li** (✉ [xcznik124@21cn.com](mailto:xcznik124@21cn.com))

Hubei Provincial Hospital of Traditional Chinese Medicine

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## Research Article

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# Abstract

**Background:** Th17 cells induced immunosuppression plays a vital role during sepsis. Belonging to the tumor necrosis factor  $\alpha$  induced protein 8 (TNFAIP8) family, TNFAIP8 associates with different immune cell physiopathological processes, thus its underlying regulatory mechanisms on Th17 cells in the acute insult processes have not been fully elucidated.

**Result:** Sepsis was induced by cecal ligation and puncture (CLP) in the male adult C57BL/6 mice. The stable TNFAIP8 knockdown Th17 cells were established via lentiviral transfection with TNFAIP8-specific SiRNA. CCK-8 assay was conducted for measuring Th17 cell proliferation. Flow cytometric analysis was adopted for examining by flow cytometry. The p53/ p21/ MDM2 pathway was measured through western blot. As a result, high TNFAIP8 expression was related with acute insult and survival rate in septic mice. TNFAIP8 SiRNA reduced Th17 cell proliferation as well as cytokines production in vivo and in vitro. In addition, TNFAIP8 KD increased the Th17 cells apoptosis in WT and septic mice. Further, TNFAIP8 influences immune function of Th17 cell involving the p53/ p21/ MDM2 signaling. Actually, TNFAIP8 KD was suggested to regulate the up-regulation of P21 and MDM2, thereby increasing p53 protein expression during sepsis. P53 gene silencing contributed to reversing cell proliferation and apoptosis regulated by TNFAIP8 KD.

**Conclusion:** Our work concluded that TNFAIP8 affected the immune function of Th17 cells, which is mediated via the p53/ p21/ MDM2 pathway after acute insult.

## Introduction

After severe trauma and surgical operation, infection, stress and other factors can cause out of control systemic inflammatory reaction and subsequent complications [1-3]. The occurrence and development mechanism of severe burn / post-traumatic sepsis is very complex, and it is closely related to the immune dysfunction, and it is necessary to explore the mechanism of immune dysfunction in sepsis. Present studies indicate that Th17 cells secrete the signature cytokine IL-17 to induce a series of tissue reactions and are critical in immune responses to infectious agents and mediate inflammation[4-6]. So, it is important to understand the Th17 cell physiopathology to explore the sepsis-related immunoregulatory mechanisms.

Tumour necrosis factor alpha-induced protein 8 (TNFAIP8) is a kind of anti-apoptotic protein during tumor or inflammatory diseases[7,8]. Expression of TNFAIP8 transcripts is found in most human cells and tissues including bone marrow, immune cells, lung, pancreas, kidney, liver, etc., and its proteins expression is induced in response to cellular inflammation mediated by TNF $\alpha$ [9,10]. Some studies reveal that TNFAIP8 expression is higher in lymphoid tissues and in the placenta, and affects T lymphocyte polarization after CLP induced sepsis[11]. Presently, studies between TNFAIP8 and Th17 cells immune and its regulatory mechanisms are lacking.

TNFAIP8 in cells is suggested to interact with p53 to affect NSCLC proliferation and cisplatin chemoresistance thereby regulating subsequent cell proliferation and cisplatin sensitivity[12]. This work analyzed the TNFAIP8 gene expression within Th17 cells after siRNA knockdown. As a result, the TNFAIP8 level was related to the p53/ p21/MDM2 signal transduction pathway. TNFAIP8 induced cell proliferation and inhibited cell apoptosis by regulating MDM2, p53 and p21 in vivo and in vitro. The present work was conducted aiming to understand the effect of TNFAIP8 expression on the immune function in Th17 cells in the acute insult.

## **Materials And Methods**

### **Experimental animals**

The male C57BL/6 mice (6-8 weeks old) utilized in the present work were provided by the Institute of Laboratory Animals Sciences, Chinese Academy of Medical Sciences. The Scientific Investigation Board of the Hubei Provincial Hospital of Traditional Chinese Medicine, Wuhan, China approved the experimental manipulations. Each experiment was performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The study was carried out in compliance with the ARRIVE guidelines (<https://arriveguidelines.org>).

### **Animals CLP model**

The mice were raised in the individual cages within the temperature-controlled room at the 12 h/12 h photoperiod, CLP (cecal ligation at the middle, followed by puncture using a 21G (0.723 mm) needle twice) was conducted to induce experimental sepsis [13]. Mice in sham groups received similar treatment without the CLP step[14].

### **Cell isolation and purification**

Spleen tissues were collected from the normal BALB/C mice and preserved within the 5 ml RPMI 1640. Thereafter, mononuclear cells were separated through Ficoll-Paque density gradient centrifugation. Later, the anti-CD4 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were used for purifying CD4<sup>+</sup> T cells among the mononuclear cells. At last, the 20 ng/mL IL-6, 1 µg/ml plate-bound anti-CD3, 2.5 ng/mL TGF-β and 1 µg/ml anti-CD28 were used to activate the naïve CD4<sup>+</sup> T cells for Th17 conditions.

### **Flow cytometric analysis for cell apoptosis**

After harvesting Th17 cells, the Annexin V buffer (BD Biosciences, San Jose, CA, USA) was adopted for washing cells, whereas Annexin V was used to stain the cells. Then, cells were stained with FITC anti-mouse IL-17 as well as APC anti-mouse CD4 antibodies (BD Biosciences, San Jose, CA, USA) for another 1 h. After measuring cell apoptosis, the 5 µg/ml 7-AAD was added. Finally, flow cytometry (BD Biosciences, San Jose, CA, USA) was conducted to analyze cell apoptosis.

### **Western blotting**

Protein concentrations were stained with anti- P53, mdm2 and p21 antibody (BD Biosciences, San Jose, CA, USA) detected through the use of the BCA Protein Assay Kit (Thermo Scientific, Grand Island, New York). Thereafter, SDS-PAGE was conducted to separate proteins, followed by electrical transfer to the PVDF membranes. Specific antibodies were utilized to probe the membranes. Besides, blots were detected through using the Pierce ECL Western blotting substrate (Thermo Scientific).

### **Real-Time Quantitative PCR (qPCR)**

The RNAeasy kit (Qiagen, Valencia, CA, USA) was adopted to extract total cellular RNA of Th17 cells. In addition, the NanoDrop 2000 spectrophotometer (Thermo, Wilmington, DE, USA) was used to quantify RNA content, whereas Fast SYBR Green Master Mix (Applied Biosystems) was adopted for quantifying DNA. In this study, the specific primer sequences (TNFAIP8) were shown below: 5'-TGAAGATGGAGCACTGCTGA-3' (forward) and 5'-GGTCTGTTACCCGTTAGGAAG-3' (reverse). At the same time, conditions for thermal cycling were shown below, 15 min under 95 °C; 40 s of denaturation under 94°C, 40 s of annealing under 50 °C and 30 s of amplification under 72°C for 36 cycles; followed by 5 min of final extension under 72°C.

### **RNA Interference**

The small interfering RNA (siRNA) was prepared by Genchem Co., Shanghai, China. For siRNA TNFAIP8, its DNA target sequence was 5'-CCG GCA TGG AGA AGT TCA AGA AGA ATT CAA GAG ATT CTT CTT GAA CTT CTC CAT GTT TTT-3'. 3'. To knock down TNFAIP8 expression, the TNFAIP8 siRNA-loaded recombinant lentiviruses were transfected into Th17 cells in Lentiviral Vector Particle in line with specific protocols.

### **Cell viability assay**

Th17 cells ( $1 \times 10^5$ /well) were grown within the 96-well flat bottom plates, cultured within the medium that contained 10% FCS and incubated within the humid incubator under 37°C and 5% CO<sub>2</sub> conditions for 24 h. Later, 10 µl CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added into each well, and a microplate reader was used to measure the absorbance (OD) value to determine the T cell proliferation ability.

### **Cytokine measurement by ELISA**

The contents of IL-17, IL-6 and IL-22 were determined by adopting the commercial ELISA kits in accordance with specific protocols (R & D Systems, Minneapolis, MN).

### **Statistics**

Each experiment was carried out thrice. The results were presented in a manner of mean±SD. ANOVA was used to examine the datasets, while different groups were compared by student's t-test. P<0.05 indicated that a difference was of statistical significance.

# Results

## TNFAIP8 mRNA and protein expressions level

To determine TNFAIP8 gene expression, total RNA of Th17 cells was extracted and RT-PCR was examined. We could note the RNA expression as expected (Fig. 1A). Expression of TNFAIP8 mRNA in Th17 cells was analyzed, and macrophages served as the positive controls. For better confirming TNFAIP8 expression, the specific TNFAIP8 Ab was used and Western blot analysis was detected. As a result, a distinct band about 21 kDa in molecular weight was observed in macrophages or Th17 cells, with macrophages being the positive controls (Fig. 1A). The results demonstrated that TNFAIP8 was expressed in Th17 cells. Further, TNFAIP8 protein levels in Th17 cells were performed at 24 h after CLP. It was presented in Figure 1B that, the TNFAIP8 levels were evidently up-regulated in CLP mice relative to sham controls ( $P < .01$ ).

## TNFAIP8 promotes Th17 cell proliferation

Since Th17 cells expressed TNFAIP8, we examined whether TNFAIP8 might be associated with Th17 cell activity during sepsis. In this work, the siRNA-mediated TNFAIP8 KD was adopted. It was shown that protein level of TNFAIP8 was significantly decreased in TNFAIP8 KD Th17 cells relative to normal controls (Fig. 2A,B,  $P < .05$ ). Then, a decrease in proliferation capacity was found in the presence of HMGB1(100ng/ml), thus, silencing TNFAIP8 gene evidently declined the proliferative response (Fig. 2C,  $P < .01$ ). At 24 h following CLP, Th17 cell proliferation in siRNA- TNFAIP8 group evidently declined relative to CLP group ( $P < .05$ ; Figure 2D).

Th17 cells have been identified to generate IL-6, IL-17 along with IL-22. Therefore, we measured the above cytokines through ELISA for identifying Th17 cell polarization. According to Fig. 2E, Th17 cells could reduce IL-17, IL-6 and IL-22 levels when stimulated with HMGB1. Besides, the contents of the above three cytokines significantly decreased after TNFAIP8 gene silenced in Th17 cells ( $P < .05$ ). Further, in vivo, the contents of the above three cytokines evidently declined in siRNA- TNFAIP8 group after CLP injury( $P < .05$ ; Figure 2F). To sum up, the above findings suggested the efficient effect of TNFAIP8 on promoting the polarization of Th17 cells.

## TNFAIP8 inhibited Th17 cell apoptosis

The Th17 cells apoptotic rate was detected by flow cytometric analysis. As shown in Fig. 3A, a significant apoptosis in the Th17 cells was promoted by HMGB1 (100 ng/ml) stimulation ( $P < .05$ ), whereas a large apoptosis within TNFAIP8 siRNA-expressed cells was observed ( $P < .05$ ). Similarly, there was much more apoptosis in the siRNA- TNFAIP8 group at 24h after CLP injury than that of CLP group( $P < .05$ ; Figure 3B).

## TNFAIP8 regulates the P53/mdm2/p21

It is well known that transcription activity is possessed during p53 up-regulation, suggesting by the elevated P53 targeted gene mRNA level, including p21 and mdm2. As observed from Fig. 4A, the levels of

p21, P53 and mdm2 increased after 24 h of incubation with 100 ng/ml HMGB1 relative to normal controls (all  $P < .05$ ), and the P53/mdm2/p21 protein levels in the TNFAIP8 KD group were strongly elevated (all  $P < .05$ ). Further, P53/mdm2/p21 levels in splenic Th17 cells after CLP injury were markedly elevated relative to sham mice ( $P < 0.01$ ), and that also evidently increased within TNFAIP8 KD mice subjected to CLP ( $P < .01$ ; Fig.4B)

### **p53 plays an important role in inhibiting Th17 cell proliferation induced by TNFAIP8 knockdown**

p53 plays an important role in inhibiting Th17 cell proliferation induced by TNFAIP8 knockdown

p53 protein controls cell DNA repair in the meantime of inducing apoptosis. p53 inhibitor Nutlin-3a was used in this experiment. The mRNA and P53 protein expression level was decreased in Th17 cells transfected with shTNFAIP8, but was significantly decreased in shTNFAIP8 + Nutlin-3a group (Figure 5A). It is shown that a decrease in proliferation capacity was found in the presence of TNFAIP8 knockdown, thus, the proliferative activity was markedly enhanced after TNFAIP8 gene knockdown and p53 inhibitor (Fig. 5B,  $P < .01$ ). Moreover, the Th17 cell apoptosis was markedly upregulated in siRNA-TNFAIP8 group in comparison with CLP group ( $P < .05$ ), whereas, the apoptosis in the Th17 cells was decreased in TNFAIP8-siRNA +p53 inhibitor group ( $P < .05$ ; Figure 5C).

## **Discussion**

TNFAIP8 is a newly identified apoptosis regulator during tumor or inflammatory diseases. As a result, the expression of TNFAIP8 was detected within the splenic Th17 cells, upregulated from septic mice and examined that the elevated levels of TNFAIP8 is related with survival rate organ injury. TNFAIP8 up-regulation in Th17 cells was possibly an indicator that predicted dismal prognosis for sepsis. In the previous study, two months after TNFAIP8L2 gene knockout, some mice developed chronic diseases, such as weight loss, splenomegaly, leukocytosis and multiple organ inflammation. After 11 months, half of the mice died of these chronic diseases, indicating that TNFAIP8L2 deficiency is easy to induce inflammation[15-18]. The above results suggested the potential of TNFAIP8 as the new marker for sepsis and inflammatory diseases.

In vitro, the immune organs and lymphoid tissues could highly express TNFAIP8, and it was demonstrated TNFAIP8 is related with the splenic T lymphocyte immune response during the development of CLP-induced sepsis[19]. It was found that Th17 cells play an important role in numerous biological processes such as inflammation, damage, infection, tumor persistence or progression. Our results verified the expression of TNFAIP8 within Th17 cells; besides, the TNFAIP8 gene was found to promote Th17 cell growth and inhibited cell apoptosis in vitro.

As suggested by our biological results, TNFAIP8 enhanced the proliferation of Th17 cells depending on p53 in vitro and in vivo. Similar to the effect of TNFAIP8 on the development of human cancer, the MDM2/p53 pathway is reported to be under the control of TNFAIP8 within cancer cells [12]. Thus, reports about the association between TNFAIP8 and wild-type p53 in Th17 cell during sepsis was scarce. In

normal cells, the expression level of p53 is very low due to rapid ubiquitination degradation. Under the condition of DNA damage or oxidative stress, p53 protein can stabilize itself and rapidly accumulate through phosphorylation, and further translocate into the nucleus to play its role as a transcription factor [20,21]. p53 is a widely expressed transcription factor that exerts a vital part in immune cells. Early studies have shown that p53 and its target genes p21 and cyclin / CDKs are involved in the pathophysiological process of sepsis, and the G1 phase arrest may affect the prognosis of severe sepsis [22-24]. Further, MDM2 is also the downstream target gene of p53, which forms a negative feedback regulation mechanism to maintain cell homeostasis under physiological conditions[23,24]. Currently, we found that the HMGB1-induced mdm2 expression showed a similar trend with that of p53, suggesting that the increased p53 level was not related to mdm2 upon HMGB1 stimulation. But, the elevated mdm2 mRNA expression was related to p53 level, thereby further confirming the normal transcriptional activity of p53 up-regulation. This observation also revealed that p53 level was higher when TNFAIP8 gene knockdown, suggesting that TNFAIP8 might negatively regulates p53 stability. Consequently, mdm2 and p21 expressions significantly increased in the TNFAIP8-siRNA group in vitro and in vivo. Such results also suggested the possible effect of TNFAIP8 on down-regulating mdm2, while mdm2 showed a similar expression trend to p53, which indicated the up-regulated p53 expression in an mdm2-independent manner upon HMGB1 stimulation and TNFAIP8 KD. Nonetheless, the up-regulated mdm2 mRNA expression related to the p53 level, thus better confirming the possible effect of TNFAIP8 on the p53/p21/MDM2 pathway in the process of sepsis.

In the present study, both p53 gene and TNFAIP8 gene was knock down by SiRNA. It was found that Th17 cell proliferative activity simulated by HMGB1 or CLP injury was markedly reduced, which was promoted upon p53 inhibitor treatment within the Th 17 cells. Moreover, we examined the cytokines, such as IL-2, IL-4, IFN- $\gamma$ , as well as the activation of intranuclear NF-AT. We found that IL-2 content and NF-AT activation strongly decreased, while the IFN- $\gamma$ /IL-4 ratio within Th17 cells in treatment with p53 inhibitor was no significant changes. These results revealed that p53 potentially affected the HMGB1-induced T cell proliferation and IL-2 production.

To sum up, TNFAIP8 is tightly related to the p53 activity within Th17 cells, while TNFAIP8 protein level has a certain effect on Th17 cell proliferation and apoptosis. Findings in this work demonstrate that TNFAIP8 regulates the immune function of Th17 cell through the MDM2/P21/p53 signal transduction pathway, which induces cell proliferation after acute injury. Collectively, our results reveal that TNFAIP8 affects the immune response of Th17 cells, and that the MDM2/P21/p53-mediated signals possibly participate in such process, thus resulting in Th17 cell immune dysfunction.

## Declarations

### Ethics approval and consent to participate

The study was approved by The Scientific Investigation Board of the Hubei Provincial Hospital of Traditional Chinese Medicine, Wuhan, China. Each experiment was performed in accordance with the

National Institute of Health Guide for the Care and Use of Laboratory Animals. The study was carried out in compliance with the ARRIVE guidelines (<https://arriveguidelines.org>).

### **Consent for publication**

Not applicable

### **Availability of data and materials**

The data is available from the corresponding author upon reasonable request.

### **Competing interests**

The authors declare that they don't have any conflict and interest.

### **Funding**

None

### **Authors' contributions**

G Li and XB Cheng mainly participated in literature search, study design, writing and critical revision. M Wang and J Li mainly participated in data collection, data analysis and data interpretation. All authors read and approved the final manuscript.

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None

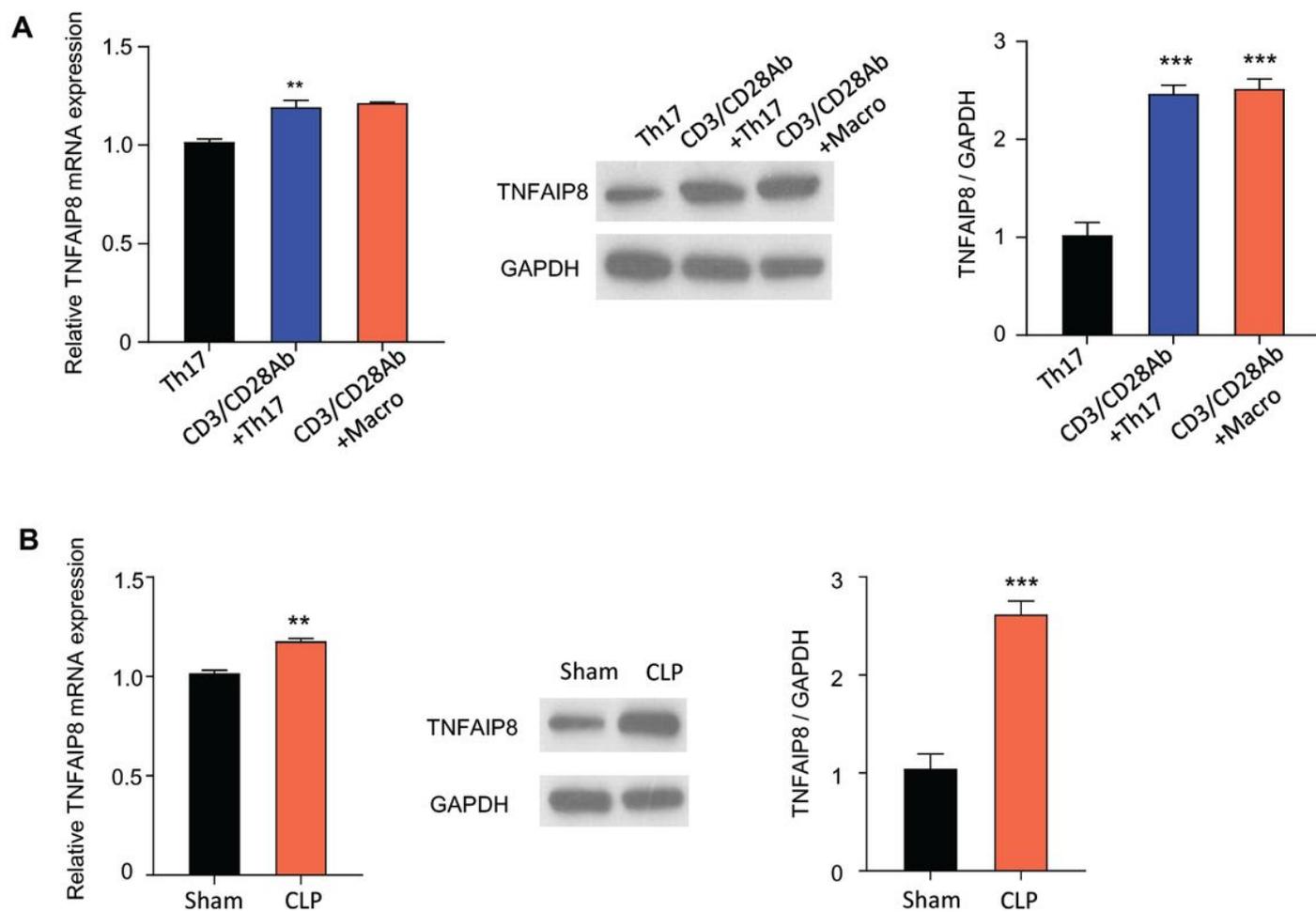
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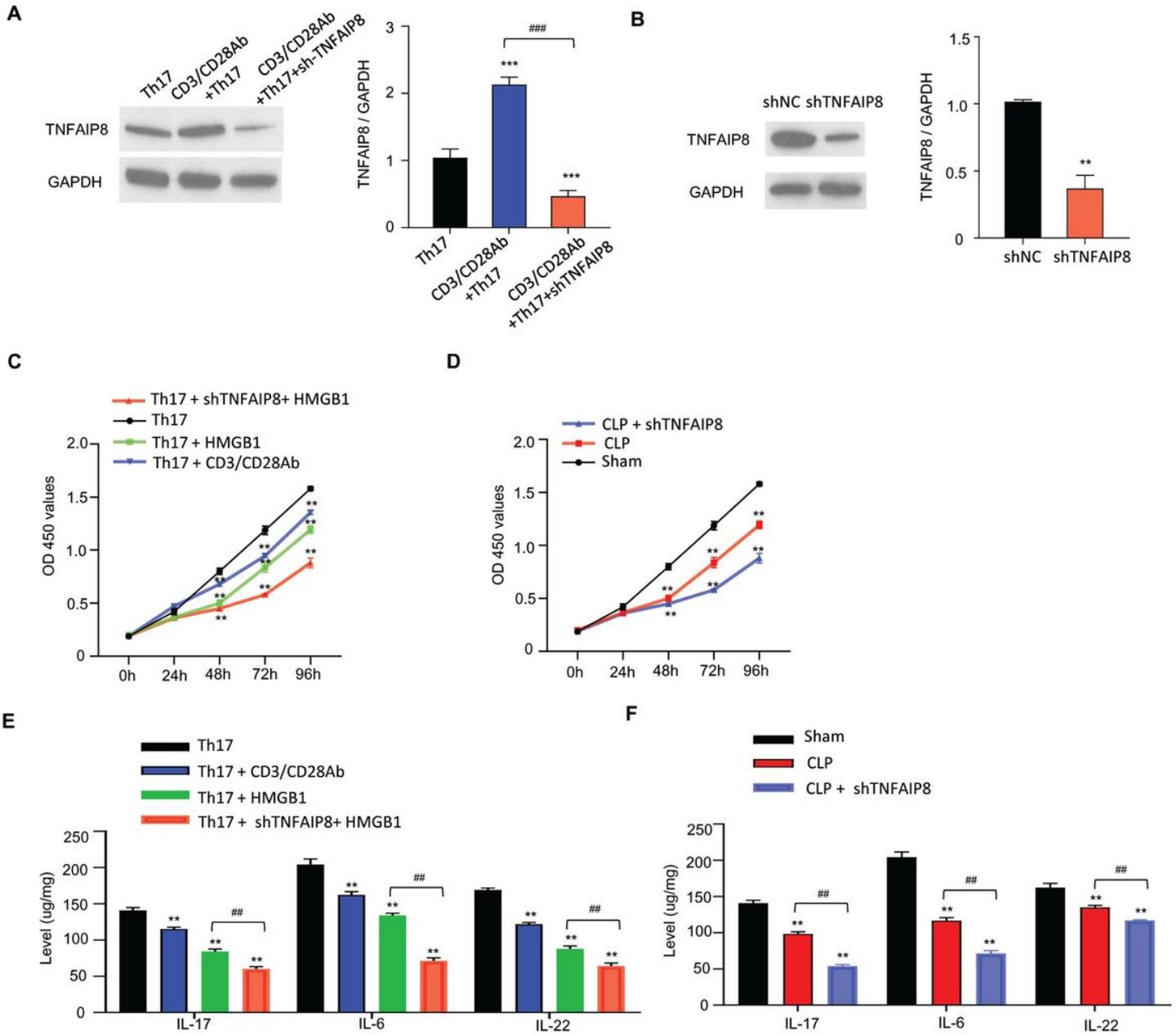
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## Figures



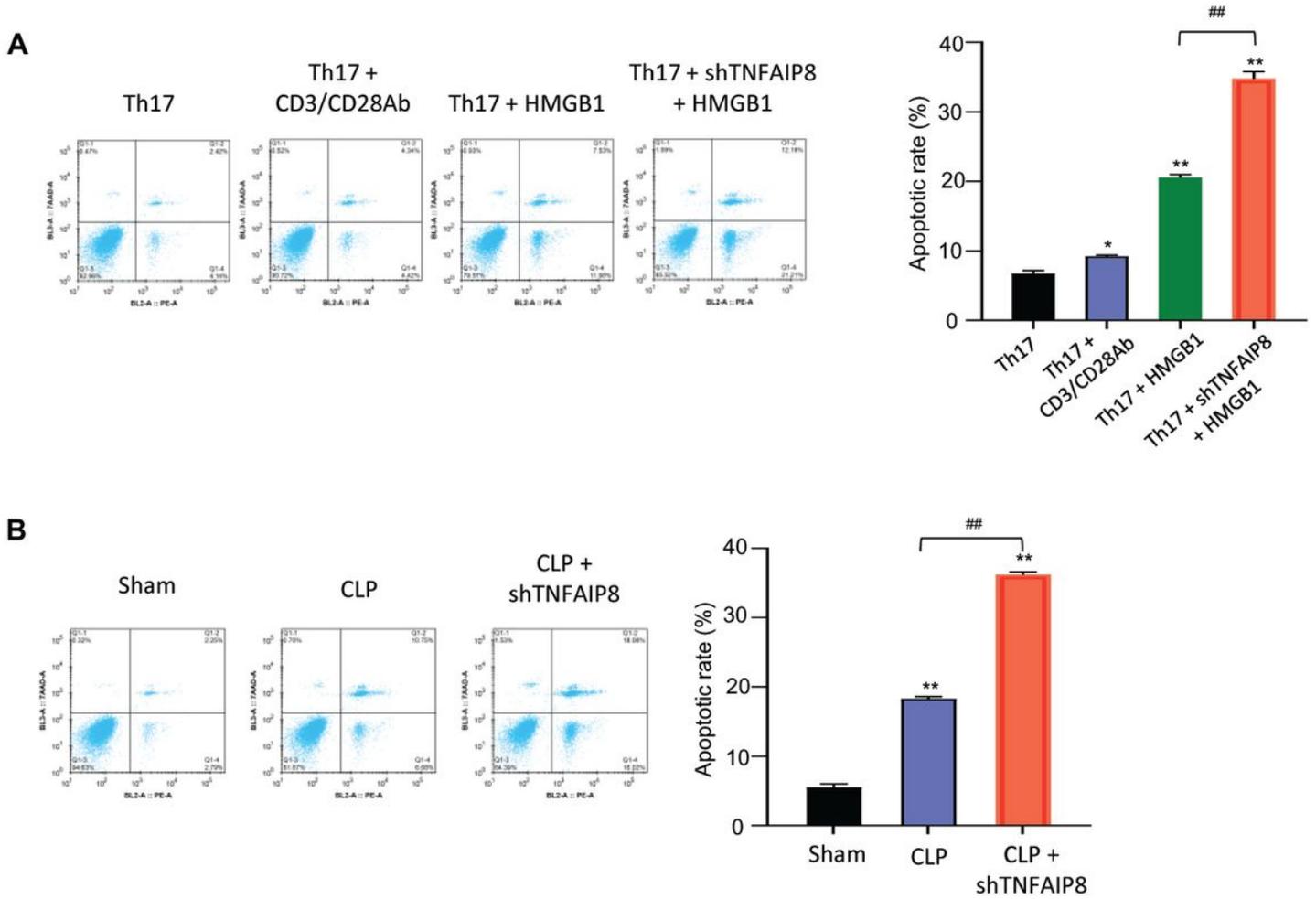
**Figure 1**

TNFAIP8 levels in Th17 cells of C57BL/6 mice (10 mice per group). (1A) The 1 µg/ml anti-CD3/anti-CD28 monoclonal Abs were used to incubate Th17 cells for 24 h. TNFAIP8 mRNA expression was detected by RT-PCR, whereas its protein expression was detected by Western blotting. (1B) In vivo, TNFAIP8 levels in Th17 cells evidently elevated relative to those of sham group and normal controls. \*P < .05



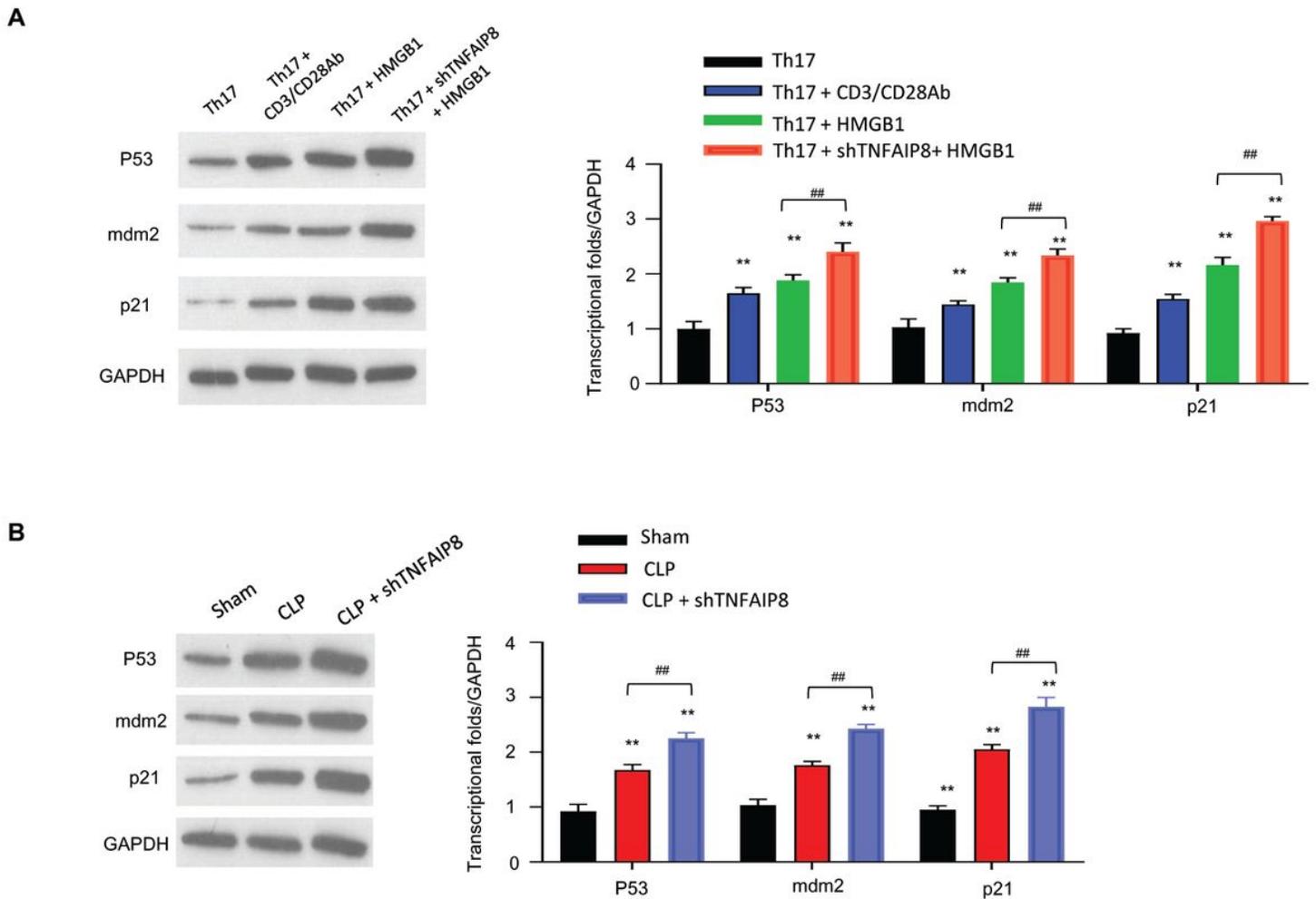
**Figure 2**

TNFAIP8 knockdown inhibits Th17 cell proliferation (10 mice per group). (2A) 1  $\mu$ g/ml anti-CD3/anti-CD28 monoclonal Abs were used to incubate Th17 cells for 24 h. TNFAIP8 protein levels in Th17 cells was shown. (2B) TNFAIP8 protein level was markedly down-regulated when TNFAIP8 gene knockdown after CLP injury. (2C) CCK-8 assay was conducted to examine Th17 cell proliferation with HMGB1 (100 ng/ml) or without HMGB1 (control) for 24 h. (2D) CCK-8 assay was conducted to examine Th17 cell proliferation at 24h after CLP. (2E) ELISA was used for measuring IL-17, IL-6, IL-22 levels in vitro. (2F) ELISA was used for measuring IL-17, IL-6, IL-22 levels in vivo. The results are presented in the manner of mean  $\pm$  SEM, \*P < .05



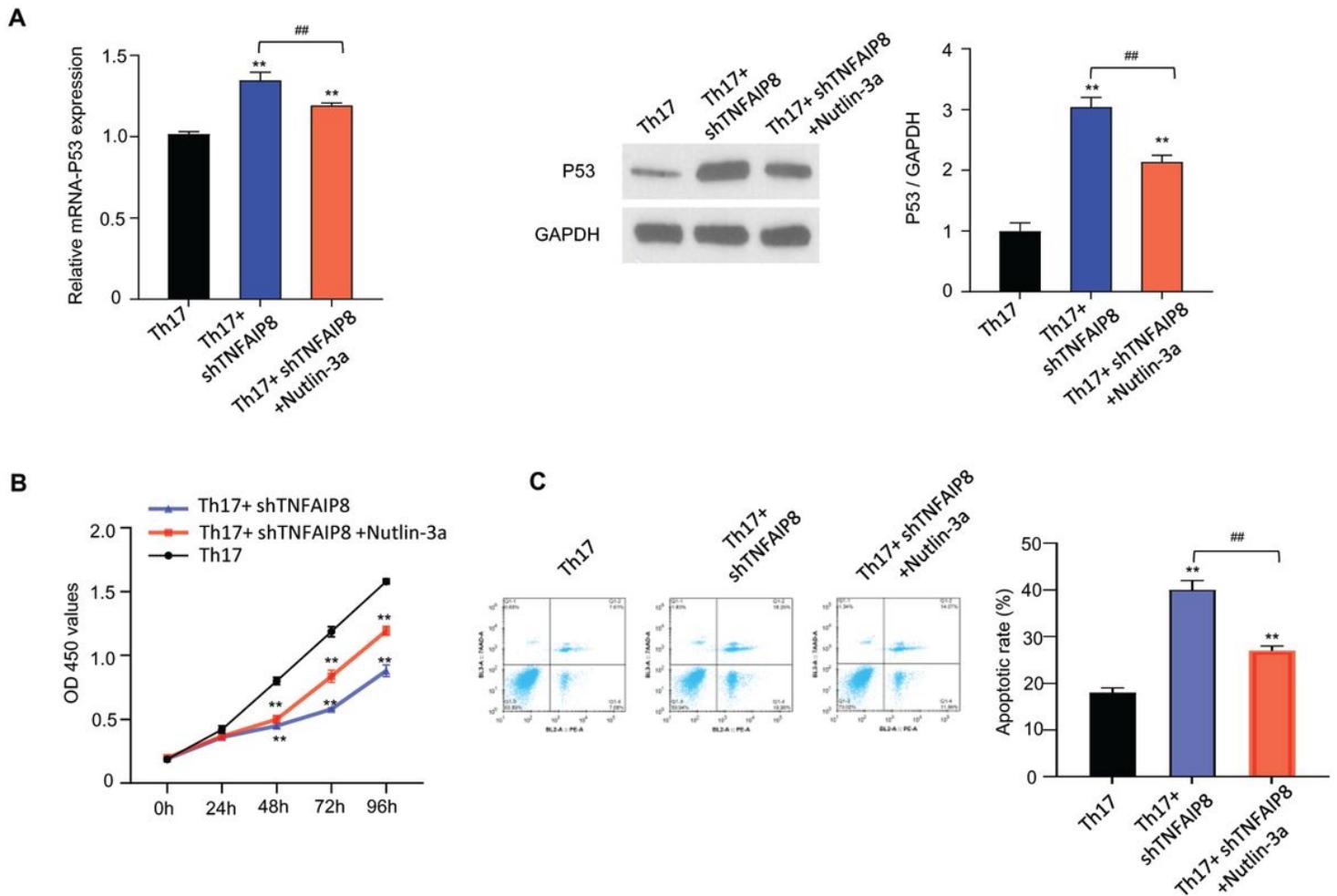
**Figure 3**

TNFAIP8 mediated Th17 cell apoptosis(10 mice per group). (3A) 1 µg/ml anti-CD3/anti-CD28 monoclonal Abs were used to incubate Th17 cells for 24 h. Flow cytometry was conducted to detect cell apoptosis. (3B) Apoptosis of Th17 cells was measured through flow cytometry at 24 h after CLP. The results are presented in the manner of mean ± SEM, \*P < 0.5



**Figure 4**

P21, mdm2 and p53 levels following TNFAIP8 knockdown(10 mice per group). (4A) 1  $\mu$ g/ml anti-CD3/anti-CD28 monoclonal Abs were used to incubate Th17 cells for 24 h. p21, p53 and mdm2 levels were detected through Western blotting. (4B) P21, p53 and mdm2 levels were detected at 24 h after CLP. The results are presented in the manner of mean  $\pm$  SEM, \*P < 0.05



**Figure 5**

p53 plays a key role in inhibiting Th17 cell proliferation induced by TNFAIP8 KD (10 mice per group). (5A) 1  $\mu$ g/ml anti-CD3/anti-CD28 monoclonal Abs were used to incubate Th17 cells for 24 h. The expression of p53 in different groups was compared using RT-PCR and Western blot. (5B) CCK-8 assay was conducted to detect Th17 cell proliferation in vitro. (5C) cell apoptosis analysis was conducted to detect Th17 cell apoptosis at 24 h after CLP. The results are presented in the manner of mean  $\pm$  SEM, \*P < 0.05