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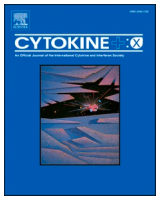

TNFAIP8 modulates the survival and immune activity of Th17 cells via p53/ p21/ MDM2 pathway after acute insult

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ABSTRACT

Th17 cells induced immunosuppression plays a vital role in sepsis. As a member of the tumor necrosis factor α induced protein 8 (TNFAIP8) family, TNFAIP8 is associated with different physiopathological conditions with immunological responses. However, its potential roles in regulating Th17 cells after the acute insult have not been fully elucidated. In this study, sepsis was induced by cecal ligation and puncture (CLP) in the male adult C57BL/6 mice. The stable TNFAIP8 knockdown (KD) Th17 cells were established by infecting with lentivirus carrying TNFAIP8-specific shRNA. CCK-8 assay was conducted to evaluate Th17 cell proliferation, and Annexin V/7-AAD assay was applied for apoptosis measurement by flow cytometry. The alterations of p53/ p21/ MDM2 pathway were assessed by Western blot. We observed that a high TNFAIP8 expression level was related to acute injury in septic mice. TNFAIP8 silencing suppressed Th17 cell proliferation and cytokine production in vivo and in vitro. In addition, TNFAIP8 KD increased Th17 cell apoptosis in septic mice. Furthermore, TNFAIP8 seems to affect the immune function of Th17 cells by regulating p53/ p21/ MDM2 signaling processes. We found that TNFAIP8 KD caused the up-regulation of P21 and MDM2, and also elevated p53 protein level during sepsis. Pharmacological inhibition of p53 partially rescued cell proliferation and apoptotic effects of TNFAIP8 KD. In summary, our work suggests that TNFAIP8 modulates the survival and immune function of Th17 cells after acute insult, which was possibly mediated through the p53/ p21/ MDM2 pathway.

Introduction

After severe trauma and surgical operation, infection, stress or other factors can cause systemic inflammatory responses and subsequent pathological complications [1–3]. The mechanism of post-traumatic sepsis development is very complex, which is closely related to immune dysfunctions of different cell types. Understanding of the immune dysregulation in sepsis is the key for immunotherapeutic intervention. Present studies indicate that Th17 cells secrete the signature cytokine IL-17 to induce a series of immune responses, which plays a critical role in immune defense against infectious agents in different inflammatory and pathological conditions [4–6]. Therefore, understanding the pathophysiological contribution of Th17 could provide novel insights into the development of sepsis-related immunotherapy.

Tumour necrosis factor alpha-induced protein 8 (TNFAIP8) is a kind of anti-apoptotic protein in tumorigenesis or inflammatory diseases [7,8]. Expression of TNFAIP8 is ubiquitously found in most human tissues, including bone marrow, immune cells, lung, pancreas, kidney, liver [9]. Its expression is induced in response to inflammatory signals mediated by TNFα [9,10]. A previous study revealed that TNFAIP8 expression is higher in lymphoid tissues and placenta, which seems to affect T lymphocyte polarization after CLP-induced sepsis [11]. Furthermore, there is evidence that intracellular TNFAIP8 interacts with p53 to affect NSCLC proliferation and cisplatin chemoresistance [12]. However, the relationship between TNFAIP8 and Th17 cells immune responses remains unclear. In the present study, we analyzed cell proliferation, apoptosis and cytokine profile in Th17 cells with TNFAIP8 silencing in sepsis mouse model. Our results showed that the TNFAIP8 was required for the survival and immune activity of Th17 in sepsis, the p53/ p21/MDM2 signal pathway seemed to contribute to the effects of TNFAIP8 on Th17 cells. Silencing TNFAIP8 induced cell apoptosis in TH17 cells and this was partially rescued by p53 inhibitor. Our work

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reveals that in sepsis TNFAIP8 modulates the survival and immune function of Th17 cells after acute insult through the p53/ p21/ MDM2 pathway.

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 protocols of animal manipulations. All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Animals CLP model

Mice were raised in individual cage within a temperature-controlled room at a 12 h/12 h dark–light cycle. CLP (cecal ligation at the middle, followed by puncture using a 21G (0.723 mm) needle) was performed 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 in RPMI 1640 medium supplemented with 10% FBS. Thereafter, mononuclear cells were purified using Ficoll-Paque density gradient centrifugation. Anti-CD4 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were used for purifying $CD4^+$ T cells among the mononuclear cells. After purification, 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 polarize the naïve $CD4^+$ T cells towards Th17 conditions. After polarization, 1 μg/ml anti-CD3/anti-CD28 monoclonal antibody was used to activate the polarized Th17 cells to mimic the Th17 activation under pathological condition. The murine macrophage cell line RAW264.7 was acquired from the cell bank of the Chinese Academy of Sciences (Shanghai, China).

Flow cytometric analysis for cell apoptosis

Cells were stained with FITC-anti-mouse IL-17 and Pacific blue-anti-mouse CD4 antibodies (BD Biosciences, San Jose, CA, USA) diluted in cold PBS with 5 % FBS for 15 mins at 4 ℃. After two times wash, apoptosis staining was performed using PE-Annexin V/7-AAD Apoptosis Detection Kit I according to the manufacturer's instructions (AB_2869265, BD Biosciences, San Jose, CA, USA). Cells were analyzed using a BD FACSARIA II flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blotting analysis

The concentration of the protein extracts from total cell lysate using RIPA buffer was determined with BCA Protein Assay Kit (Thermo Scientific, Grand Island, New York), and 10 ug total protein was loaded into SDS-PAGE gel for electrophoresis, followed by protein band transfer onto the PVDF membranes. After blocking with 5% skim milk, Anti-p53 (ab131442), MDM2 (ab16895) and p21 (ab227443) antibodies (BD Biosciences, San Jose, CA, USA) were used to detect the proteins on the blot. Protein bands were developed using the Pierce ECL Western blotting substrate (Thermo Fisher Scientific).

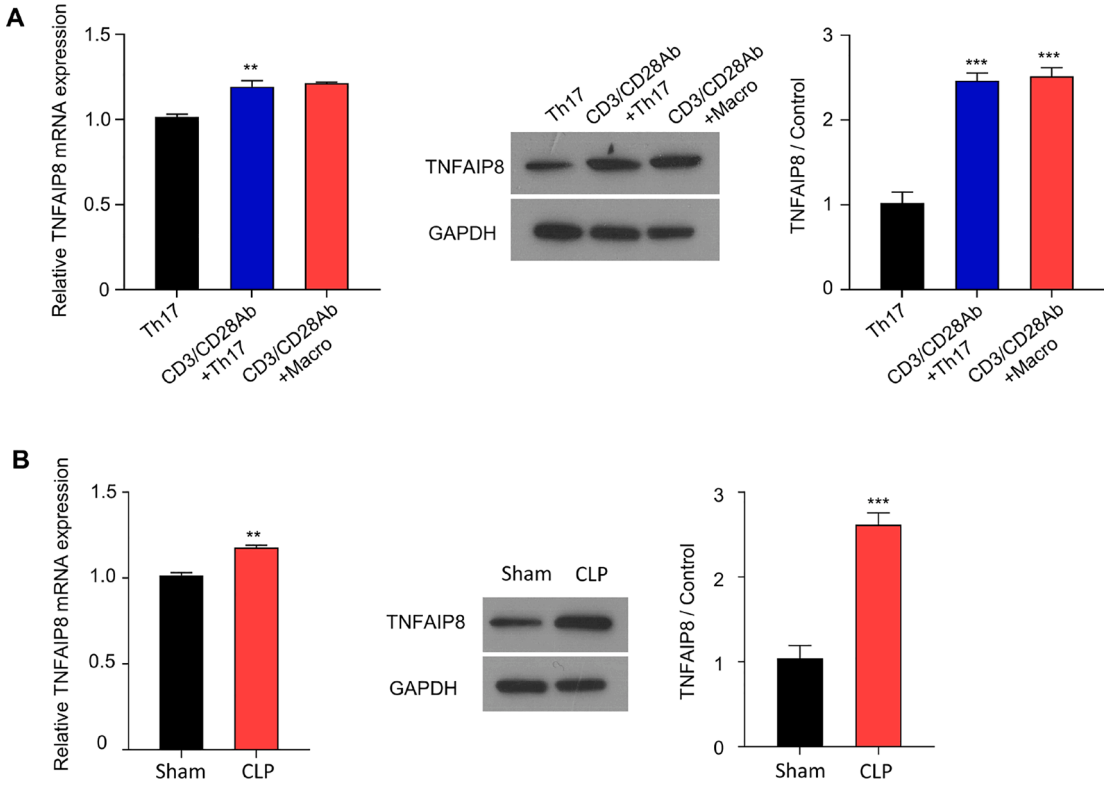

Fig. 1. TTNFAIP8 levels in activated Th17 cells and sepsis-induced C57BL/6 mice (10 mice per group). (A) Primary $CD4^+$ T cells was polarized under Th17 conditions. 1 μg/ml anti-CD3/anti-CD28 monoclonal antibody was used to activate the polarized Th17 cells and primarily isolated macrophages for 24 h. TNFAIP8 mRNA expression was detected by RT-qPCR, and the protein level was detected by Western blotting. (B) Th17 cells were isolated from mice with CLP-induced mice and the sham group. TNFAIP8 mRNA expression was detected by RT-qPCR, and the protein level was detected by Western blotting. Data are the summary of 3 independent experiment and statistical comparison was performed by two-tailed students' *t* test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

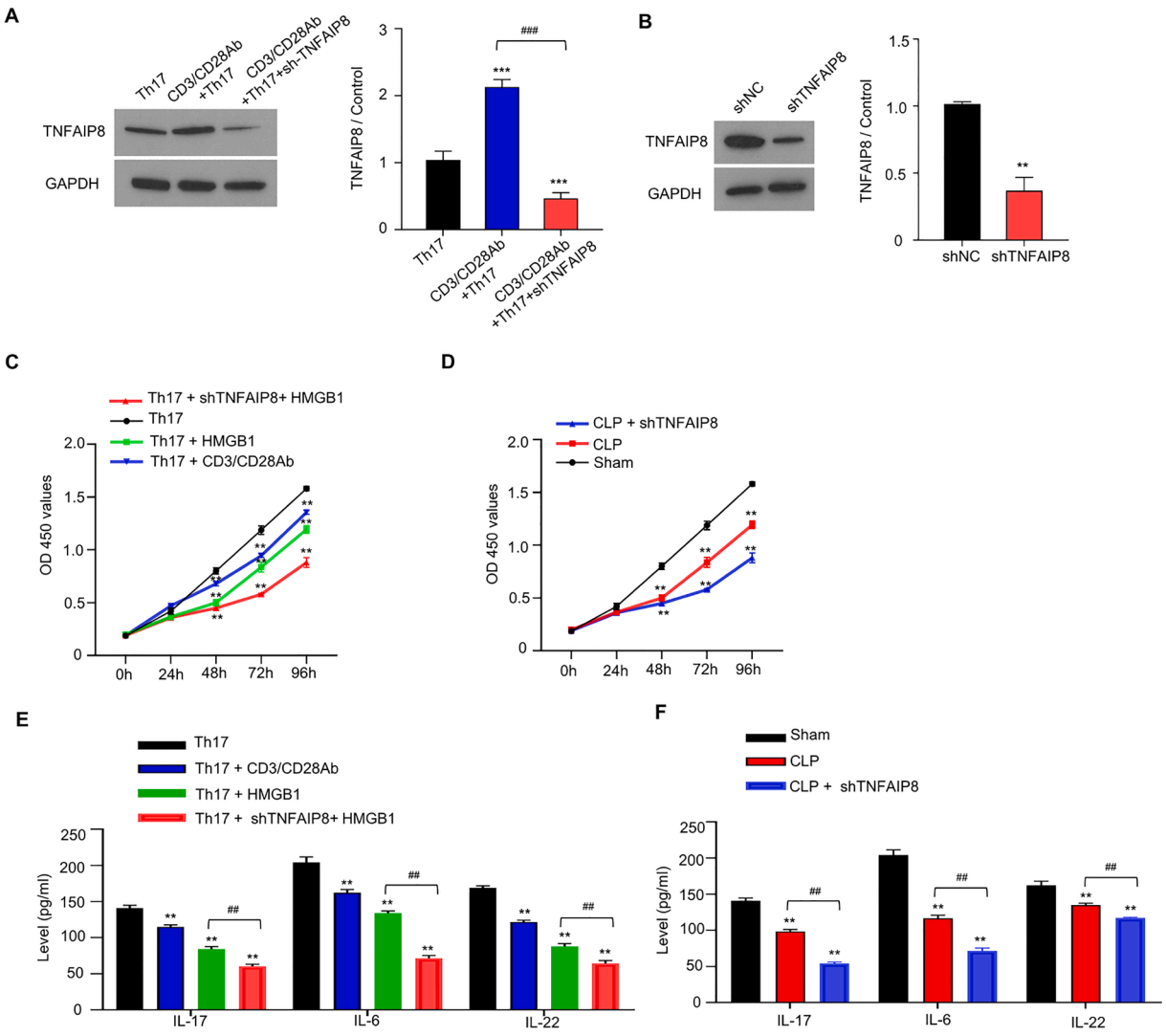

Fig. 2. TNFAIP8 knockdown inhibits Th17 cell proliferation. (A) Primary $CD4^+$ T cells was polarized under Th17 conditions. 1 μg/ml anti-CD3/anti-CD28 monoclonal antibodies were used to activate Th17 cells and Th17 cells expressing shRNA for TNFAIP8. TNFAIP8 protein levels in each condition of Th17 cells was shown and bar chart represents the quantified protein band intensity by densitometry, which was normalized to GAPDH. (B) TNFAIP8 protein level was markedly down-regulated when TNFAIP8 gene was silenced in Th17 cells from mice with CLP injury. (C) CCK-8 assay was performed to examine Th17 cell proliferation with CD3/CD28 activation, with HMGB1 (100 ng/ml) treatment, with HMGB1 treatment and TNFAIP8 silencing or without HMGB1 (control). (D) CCK-8 assay was performed to examine proliferation of Th17 cells isolated from CLP-mice, CLP mice with TNFAIP8 silencing and sham groups (10 mice per group). (E) ELISA measurement of the concentration of IL-17, IL-6, IL-22 in the cell culture medium described in (C). (F) ELISA measurement of the concentration of IL-17, IL-6, IL-22 in Th17 cell culture media of conditions described in (D). The results were summary of mean ± SEM. Two-Way ANOVA plus Bonferroni post-hoc test was used to examine the datasets with two categorical variables, while comparisons between two groups were compared by student's *t*-test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Real-time quantitative PCR (qPCR)

The RNeasy Mini kit (Qiagen, Valencia, CA, USA) was used to extract total cellular RNA from Th17 cells. A NanoDrop 2000 spectrophotometer (Thermo, Wilmington, DE, USA) was used to quantify RNA concentration. 5 μg total RNA was covered to cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Fast SYBR Green Master Mix (Applied Biosystems) was used for real-time qPCR on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). The primer sequences (TNFAIP8) used in this study were shown below: Forward primer 5′-TGAAGATGGAGCACTGCTGA-3′ and reverse primer: 5′-GGTCTGTTACCCGTTAGGAAG-3′. Conditions for thermal cycling: 5 min under 95 °C; 40 cycles of 30 sec of denaturation at 94 °C and 60 sec of annealing and extension at 60 °C, signal was detected at the end of each cycle.

Stable shRNA silencing

The lentiviral particles containing shRNA targeting TNFAIP8 was prepared by Genchem Co., Shanghai, China. The sequence corresponding to TNFAIP8 shRNA is 5′-CCG GCA TGG AGA AGT TCA AGA AGA ATT CAA GAG ATT CTT CTT GAA CTT CTC CAT GTT TTT-3′. Th17 cells was infected with TNFAIP8 shRNA containing recombinant lentiviruses according to the manufacture's instruction.

Cell proliferation assay

Th17 cells (1×10^5/well) were seeded into a 96-well flat bottom plates cultured within the RPMI 1640 medium that contained 10% FCS in a humid incubator at 37 °C and 5% CO2 conditions for 24 h. 10 μl CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added into each well for 1-hour incubation and a synergy HTX

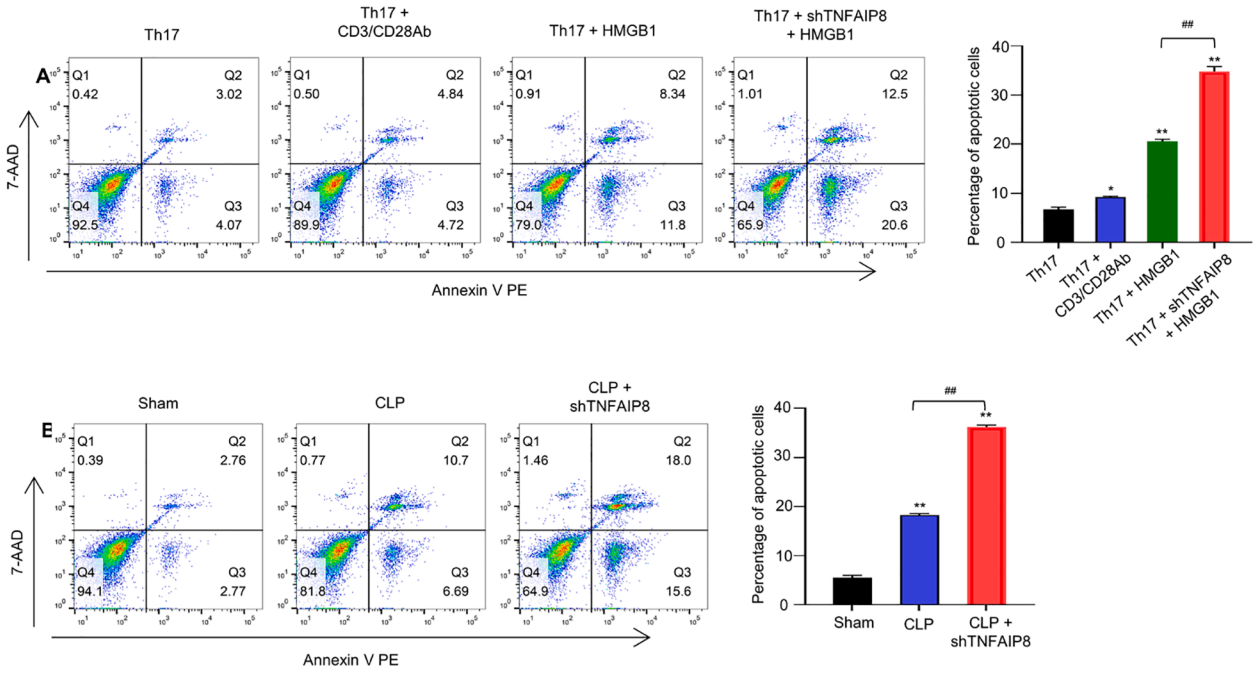

Fig. 3. TNFAIP8 mediates Th17 cell apoptosis. (A) Primary CD4+ T cells was polarized under Th17 conditions. 1 μg/ml anti-CD3/anti-CD28 monoclonal antibodies were used to activate Th17 cells and Th17 cells expressing shRNA for TNFAIP8. Annexin V and 7-AAD staining assay was used to analyze apoptosis using flow cytometry. (B) Apoptosis of measured in Th17 cells isolated from CLP-mice, CLP mice with TNFAIP8 silencing and sham groups (10 mice per group) 24 h after CLP. The results were summary of mean ± SEM. Comparisons between two groups were compared by student's *t*-test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

multi-mode microplate reader (Biotek) was used to measure the absorbance (OD) value to determine the T cell proliferation ability.

Cytokine measurement by ELISA

Th17 cells (1 × 105/well) were seeded into a 96-well flat bottom plates cultured within the RPMI 1640 medium for 24 h. 100 ul of cell culture supernatnt was used for ELISA. The contents of IL-17, IL-6 and IL-22 were determined by the commercial ELISA kits in accordance with specific protocols (R & D Systems, Minneapolis, MN), using a synergy HTX multi-mode microplate reader (Biotek).

Statistical analysis

Each experiment was carried out three times. The results were presented as mean ± S.D. Two-Way ANOVA plus Bonferroni post-hoc test was used to examine the datasets with two categorical variables, while comparisons between two groups were compared by student's *t*-test. Data with a $P < 0.05$ was considered as statistically significant.

Results

TNFAIP8 mRNA and protein expression level increases in Th 17 cells after in vitro activation or in CLP model

High expression of TNFAIP8 in macrophage RAW264.7 has been previously confirmed in literature [15] and RAW264.7 was therefore used as positive control in detection the TNFAIP8 gene expression in cells. 1 μg/ml anti-CD3/anti-CD28 monoclonal Abs were used to activate Th17 cells and macrophages for 24 h, which mimic the cell activation under pathological condition. TNFAIP8 mRNA expression in Th17 cells was found to be significantly increased after activation (Fig. 1 A). To confirm TNFAIP8 protein level, a specific TNFAIP8 Antibody was used for Western blot analysis. Consistently, TNFAIP8 protein level increased after activation (Fig. 1A). We further analyzed TNFAIP8 mRNA and protein levels in Th17 cells isolated from mice after CLP procedures. TNFAIP8 levels were evidently up-regulated in CLP mice when compared to sham controls (Fig. 1B). These data suggest that TNFAIP8 can be up-regulated after Th17 cells activation in vitro and in vivo.

TNFAIP8 is required for Th17 cell proliferation and immune functions

We next examined whether TNFAIP8 was functionally associated with Th17 cell activity during sepsis. To do this, the shRNA-mediated TNFAIP8 KD was performed to stably silencing TNFAIP8. It was shown that protein level of TNFAIP8 significantly decreased in TNFAIP8 KD Th17 cells in relative to controls (Fig. 2A-B, $P < 0.05$). We then performed CCK8 proliferation assay and found a decrease in proliferation capacity in the presence of HMGB1 (100 ng/ml). Silencing TNFAIP8 gene further inhibited the proliferative response (Fig. 2C, $P < 0.01$). At 24 h following CLP, the proliferation rate of shRNA-TNFAIP8 group also declined in relative to CLP group ($P < 0.05$; Fig. 2D).

Th17 cells can produce cytokines such as IL-6, IL-17 along with IL-22. We then measured the above cytokines using ELISA to identify its cytokine profile. According to Fig. 2E, Th17 cells showed reduced production of IL-17, IL-6 and IL-22 when stimulated with HMGB1. Besides, the contents of the above three cytokines significantly decreased in Th17 cells with TNFAIP8 silencing ($P < 0.05$). Furthermore, in the CLP model, the levels of the above cytokines evidently declined in shRNA- TNFAIP8 group after CLP injury ($P < 0.05$; Fig. 2F). In summary, the above findings suggested a functional requirement of TNFAIP8 on the proliferation and polarization of Th17 cells.

TNFAIP8 silencing leads to Th17 cell apoptosis

To investigate whether TNFAIP8 is required for the survival of Th17 cells, apoptosis assay was performed by flow cytometric analysis using Annexin V/7-AAD staining. Annexin V stains for apoptotic cells with phosphatidylserine externalization and 7-AAD serves as cell-impermeable dye to distinguish early and late apoptotic cells. The percentage of overall apoptotic cells increased by HMGB1 stimulation when

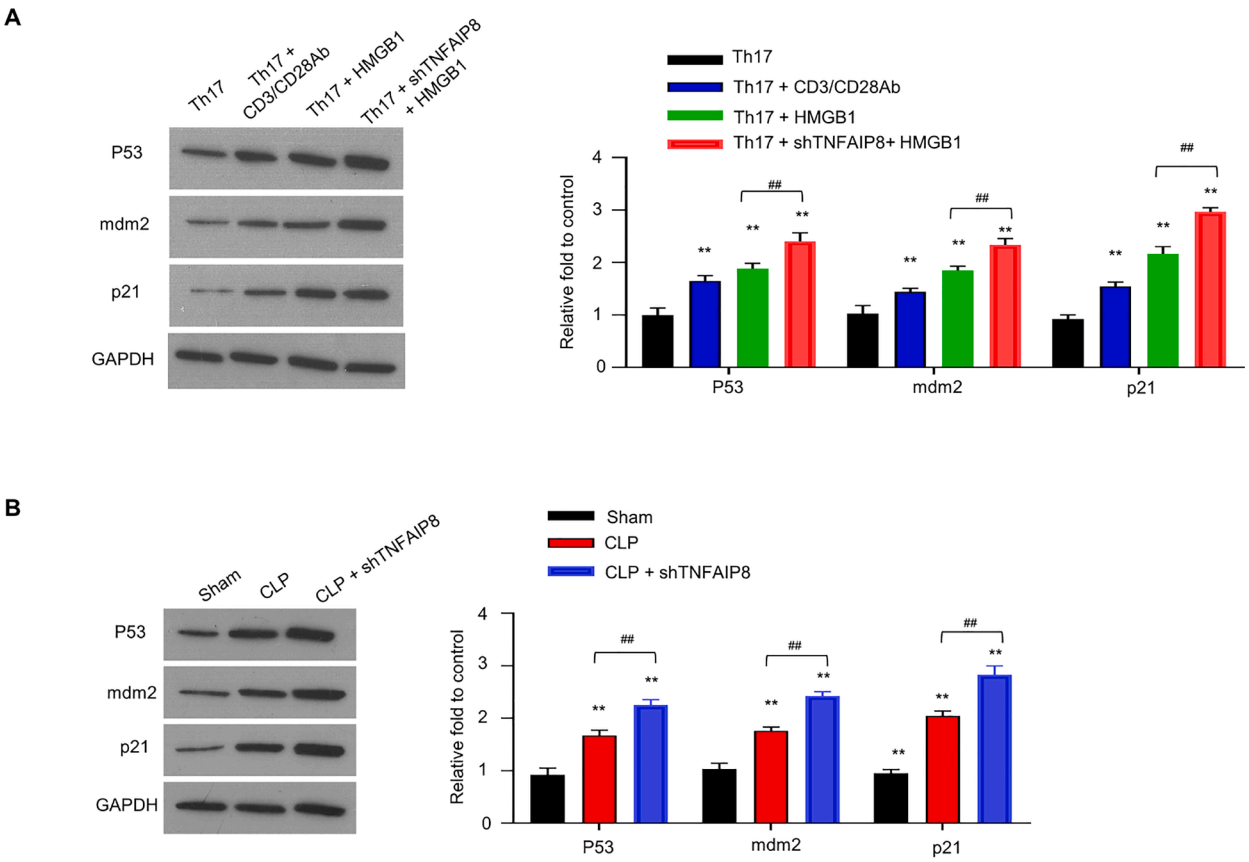

Fig. 4. TNFAIP8 silencing dysregulates the p53/MDM2/p21 signaling pathway (A) Primary CD4+ T cells was polarized under Th17 conditions. Th17 cells were treated with CD3/CD28 activation, HMGB1 (100 ng/ml) treatment, with HMGB1 treatment and TNFAIP8 silencing or without HMGB1. Western blot was used to measure the protein levels of p21, p53 and MDM2. (B) p21, p53 and MDM2 levels were detected in Th17 cells isolated from CLP-mice, CLP mice with TNFAIP8 silencing and sham groups 24 h after CLP. p21, p53 and MDM2 levels were normalized to GAPDH. The results were summary of mean ± SEM and comparisons between two groups were compared by student's *t*-test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

compared to the untreated control ($P < 0.05$), and an even higher percentage of apoptotic cells were observed in TNFAIP8-silenced cells in the presence of HMGB1 treatment ($P < 0.05$, Fig. 3A). Similarly, TNFAIP8-silenceing in Th17 cells caused a higher level of apoptosis 24 h after CLP injury ($P < 0.05$; Fig. 3B). Therefore, our data suggest that TNFAIP8 is required to maintain the survival of Th17 cells.

TNFAIP8 silencing dysregulates the p53/MDM2/p21 signaling pathway

p53 protein acts as a tumor-suppressive and pro-apoptotic gene, regulating the expression of a variety of proapoptotic genes. We examined the expression levels of p21, MDM2 and p53 after TNFAIP8 silencing. Protein levels of p21, MDM2 and p53 increased after HMGB1 treatment and TNFAIP8 silencing further elevated the levels of all the proteins (Fig. 4A). Further, p53/MDM2/p21 levels in splenic Th17 cells after CLP injury markedly increased in relative to sham mice ($P < 0.01$), and TNFAIP8 silencing further enhanced the levels of all the proteins in CLP mice ($P < 0.01$; Fig. 4B). These data reveal the activation of p53 signals after TNFAIP8 silencing.

p53 activity mediates the inhibitory effect of TNFAIP8 silencing on Th17 cell proliferation

p53 transcription activity can lead to cell cycle arrest and apoptosis. We next tested whether p53 activity is involved in the proliferation inhibition by TNFAIP8 silencing. A p53 inhibitor, Pifithrin-α (PFT-α) was used in this experiment. It was shown that a decrease in proliferation capacity was observed in the presence of TNFAIP8 silencing. However, the proliferative activity was partially rescued in the presence of p53 inhibitor treatment (Fig. 5A-B, $P < 0.01$). Moreover, p53 inhibition also decreased the percentage of apoptotic cells in TNFAIP8-silenced Th17 cells (Fig. 5C). Taken together, our data imply that the up-regulation of p53 and its activity at least partially accounts for the effect of TNFAIP8 silencing in Th17 cells.

Discussion

TNFAIP8 is a newly identified regulator of apoptosis in tumorigenesis or inflammatory diseases. Our study further demonstrated the up-regulation of TNFAIP8 in activated Th17 cells as well as in CLP-induced sepsis in mouse model. TNFAIP8 up-regulation in Th17 cells is a possible indicator of the dismal prognosis for sepsis. In the previous studies, mice developed chronic pathological conditions two months after TNFAIP8L2 gene knockout, such as weight loss, splenomegaly, leukocytosis and multiple organ inflammation [16–19]. After 11 months, half of the mice died of these chronic diseases, indicating that TNFAIP8L2 deficiency induces inflammation [16–19]. The above results suggest the potential of TNFAIP8 as a new marker for sepsis and inflammatory diseases.

The immune organs and lymphoid tissues also express high level of TNFAIP8, and it has been demonstrated that TNFAIP8 expression correlates with splenic T lymphocyte immune responses during the development of CLP-induced sepsis [20]. Previous studies that Th17 cells played an important role in numerous pathological conditions such as inflammation, damage, infection, tumor persistence or progression [4–6]. Our results further revealed that the upregulation of TNFAIP8

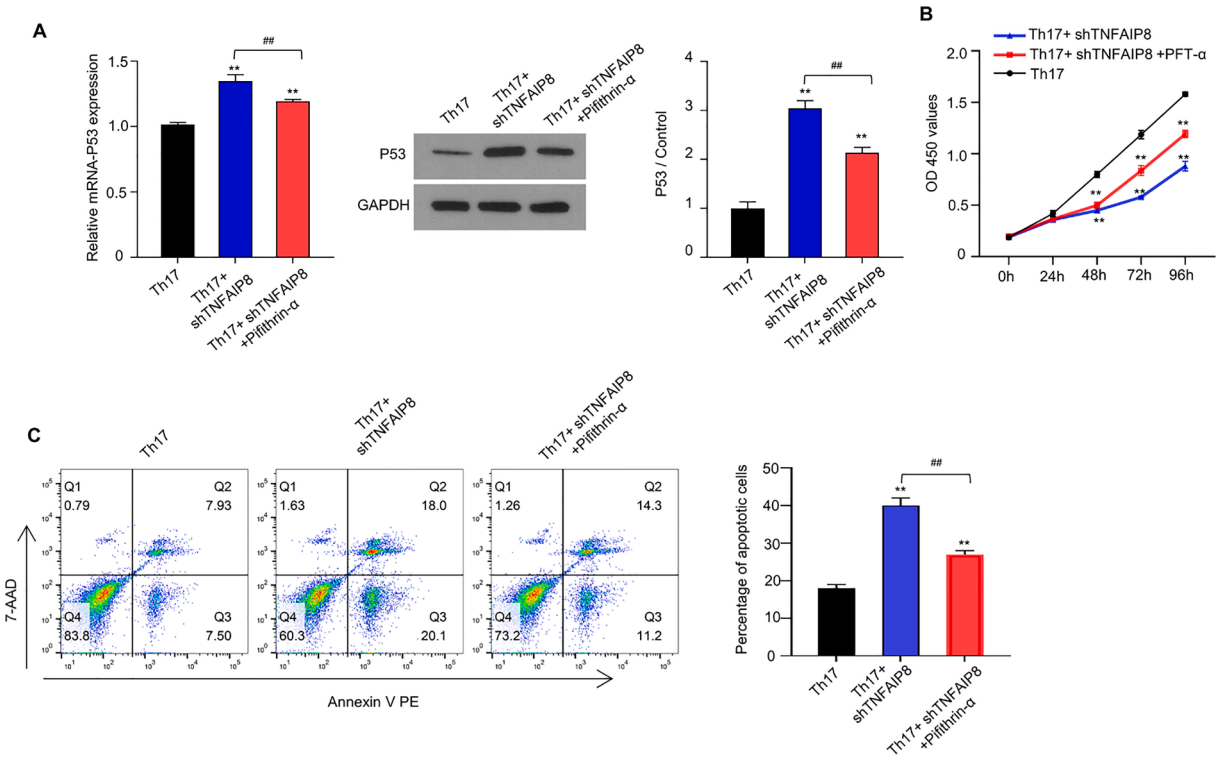

Fig. 5. p53 activity inhibition mitigates the inhibitory effect of TNFAIP8 silencing on Th17 cells. (A) p53 mRNA expression (Left bar chart) and protein level (right bar chart) was detected by RT-PCR and Western blotting respectively in Th17 cells, Th17 cells expressing TNFAIP8 shRNA, and Th17 cells expressing TNFAIP8 shRNA plus Pifithrin-α (PFT-α, 10 uM). Bar chart (right) represents the quantified protein band intensity by densitometry, which was normalized to GAPDH. (B) CCK-8 assay proliferation assays in Th17 cells described in (A). (C) Apoptotic assay of Th17 cells described in (A) was analyzed by flow cytometry. The results were summary of mean ± SEM. Two-Way ANOVA plus Bonferroni post-hoc test was used to examine the datasets with two categorical variables, while comparisons between two groups were compared by student's *t*-test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

within activated Th17 cells and Th17 cells from septic mice. Besides, TNFAIP8 seems to be necessary for the maintenance of Th17 cell growth and immune responses.

As suggested by our results, the effect of TNFAIP8 deficiency in Th17 cells depends on p53 activity in vitro and in vivo. Consistent with the effect of TNFAIP8 on the development of human cancer, MDM2/p53 pathway is reported to be under the control of TNFAIP8 within cancer cells [12]. In normal cells, the expression level of p53 is very low due to rapid ubiquitination-dependent degradation. Under the condition of DNA damage or oxidative stress, p53 protein becomes stabilized and rapidly accumulates through phosphorylation, and its translocation into the nucleus regulates key genes involved in DNA damage or stress responses [21,22]. p53 is also widely expressed in different types of 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 of target cells may affect the prognosis of severe sepsis [23–25]. Further, MDM2 is also the downstream target gene of p53, which forms a negative feedback regulation mechanism to maintain the homeostasis of p53 protein under physiological conditions [24,25]. Recent studies found that the HMGB1-induced MDM2 expression showed a similar trend with that of p53, suggesting that the increased p53 level was due to MDM2-dependent degradation upon HMGB1 stimulation [26]. However, the elevated MDM2 expression seems to be dependent on p53 level, thereby confirming negative feedback regulation of p53 activation. In this study, we further revealed that p53 level was higher upon TNFAIP8 gene knockdown, suggesting that TNFAIP8 might negatively regulate p53 stability in Th17 cells. Consequently, MDM2 and p21 expression was also augmented in TNFAIP8-silenced Th17 cells. Our data collectively suggest that TNFAIP8 negatively impacts on p53 level in a MDM2 independent manner, which in turns affects the downstream genes such as MDM2 and p21. Nonetheless, the direct dependence of MDM2 and p21 mRNA expression on up-regulated p53 level in TNFAIP8 silenced Th 17 cells needs to be further validated in the sepsis.

In summary, TNFAIP8 expression level is tightly related to the p53 protein level and activity within Th17 cells, which altogether effects the proliferation, apoptosis and immune functions of Th17 cells. Whether the immune functions of Th17 cell modulated by TNFAIP8 are regulated by MDM2/p21/p53 signal transduction pathway will require further investigation. Collectively, our results reveal a novel role of TNFAIP8 in regulating the survival and immune responses of Th17 cells, and suggest that targeting p53 activity can potentially improve Th17 cell survival and immune functions in sepsis.

Authors' contributions

G Li and XB Cheng mainly participated in literature search, study design, manuscript writing and critical revision. M Wang and J Li mainly participated in data collection, data analysis and data interpretation. Mr. Xiaocheng Shen re-analyzed the flow cytometry data, revised the paper and drafted the response letter. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Xiaobin Cheng: Data curation, Writing – original draft. **Xiaocheng Shen:** Software, Writing – review & editing. **Min Wang:** Visualization, Investigation. **Jing Li:** Software, Validation. **Gang Li:** Conceptualization, Methodology, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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