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TRAF3 plays a role in lupus nephritis by regulating Th17 cell and Treg cell balance as well as NF-κB signaling pathway in mice

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Abstract. Lupus nephritis (LN) occurs with inflammatory lesion in patients suffering from systemic lupus erythematosus (SLE). Tumor necrosis factor (TNF) receptor associated factor 3 (TRAF3) is an important mediator in inflammation. To explore the roles of TRAF3 in LN, the LN mouse model was firstly established with intraperitoneal (i.p.) injection of pristine. Our results found that the amount of urinary protein was increased evidently at day 28, and renal damage occurred in the LN mouse model, but the TRAF3 knockdown reduced the urinary protein and alleviated the inflammatory lesion. The pro-inflammatory cytokines TNF-α, IL-1β, IL-17a, IFN-γ and IgM, IgG antibody were enriched, but there was little amount of IL-10 in the LN mouse model. Moreover, the amount of $CD40^+$ B cells, $CD4^+$ T cells sub-type, Th17 cells were abundant, and the proteins TRAF3, TRAF2, NF-κBp52, IKKα, ICAM1 in the kidney were highly expressed in the LN mouse model. However, TRAF3 knockdown enhanced the production of IL-10 and reduced the amount of pro-inflammatory cytokines, immunoglobulin, and the protein expressions of TRAF3, TRAF2, NF-κBp52, IKKα, ICAM1. In conclusion, TRAF3 plays a role in LN by regulating Th17 cell and Treg cell balance as well as NF-κB signaling pathway in mice.

Key words: Lupus nephritis — TRAF3 — $CD40^+$ B cells — Th17 cells — NF-κB signaling pathway

Abbreviations: BAFF, B cell-activating factor; DCs, dendritic cells; LN, lupus nephritis; NIK, NF-κB inducing kinase; NK, natural killer; shRNAs, short hairpin RNAs; SLE, systemic lupus erythematosus; TNF, tumor necrosis factor; TRAFs, receptor associated factors; TRAF3, receptor associated factor 3.

Introduction

Systemic lupus erythematosus (SLE) is a kind of autoimmune disease, which induced serious damage to multiple organs (Durcan et al. 2019). Lupus nephritis (LN) is a common complication of SLE, and it's estimated that approximately 30–60% of adults and up to 70% of children with SLE suffered from kidney damage (Davidson 2016; Chang et al. 2021).The pathogenesis of LN mainly is the infiltration of inflammatory cells and the formation of immunocomplex, which induce the inflammatory damage in kidney (Hong et al. 2020). The study of Arazi et al. (2019) revealed 21 immune cell sub-types in LN, such as the clusters of T cells, natural killer (NK) cells, dendritic cells (DCs) and B cells. Besides, with the single-cell RNA sequencing (scRNA-seq) analysis, the cell sub-types of $CD8^+$ T cells, $CD4^+$ T cells, B cells, monocyte and myeloid populations were also found

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to be infiltrated in the kidney (Rao et al. 2020). Moreover, the immunoglobulin G (IgG), IgM and auto-antibodies (anti-DNA and anti-Sm) increased in the constructed SLE mouse model of Yang-Yang He' research (He et al. 2016). However, the molecular mechanism of LN is still remained unclear despite numerous clinical and animal studies. Hence, it is of great importance to reveal the mechanism of inflammatory damage in LN.

The tumor necrosis factor (TNF) receptor associated factors (TRAFs) are a family of proteins (TRAF1-TRAF7) participated in cell survival, apoptosis and immune inflammatory responses (Swaidani et al. 2019; Li et al. 2020). TRAF3 have been found to be an important regulator of B cell-activating factor (BAFF), CD40, lymphotoxin β receptor, etc in anti-inflammation and autoimmune response (Xu and Shu 2002; Wimmer et al. 2013). TRAF5 and TRAF2 share an overlapping binding site of TRAF3 in the NF-κB inducing kinase (NIK) degradation process (Sajjad et al. 2019). Moreover, TRAF3 could interact with Act1-mediated autoimmune diseases (i.e. Sjogren's disease, LN) through noncanonical NF-κB activation pathway (Li 2008). Hence, our study is aimed to study the effect of TRAF3 on the induction of LN disease.

Materials and Methods

Plasmids construction

The short hairpin RNAs (shRNAs) of the mouse TRAF3 gene (NCBI accession number: NM_001364364.1) were designed by Guangzhou RiboBio Co., Ltd (No. siG171027014746, Guangzhou, China). Three pairs of shRNAs were cotransfected into 293T cells (No. A1662, Mcellbank, China) with Lipofectamine 2000 (No. 11668019, Invitrogen, USA) to screen the optimal shRNA. Afterwards, the optimal shRNA sequence against mouse TRAF3 gene ('5-CACGACAGAGCTGCTGAGTACTTTA-3') was cloned into the pENTR-GFP plasmid (No. LM60908-2740, LMAI Bio, China). The recombinant plasmid was transfected into Escherichia coli DH5a (No. A0659, EK-Bioscience, China), and the stable transfected plasmid was screened with G418 (No. PB180125, Procell, China). Besides, the sh-control plasmid was constructed with the same protocol. Importantly, the plasmid and transfection agents were diluted, mixed and shaken according to the protocol.

LN mouse model establishment

The BALB/c female mice (age 8–9 weeks) (n = 60) were obtained from Beijing Vital River. They were housed in pathogen-free conditions on a 12-h light/dark cycle for 10 days adaptation. Afterwards, the mice were divided into four groups (n = 15 in each); the mice, intraperitoneally (i.p.) injected with 500 μl vehicle with 0.9% saline, were set as the normal control (NC group), and the rest mice (n = 45 in total) were received 500 μl pristane (No. P106824, Sigma-Aldrich, USA) by i.p. injection to construct the LN mouse model (Model group) (Zhou et al. 2020). Meanwhile, two groups of the LN mouse model were respectively received control shRNA interference plasmid (sh-control group) and TRAF3 shRNA interference plasmid (sh-TRAF3 group) by tail vein injection. Besides, the other two groups mice were tail vein injected with the same amount of saline. The grouping and operations are shown in Table 1. Importantly, all animal experimental procedures were approved by the Ethic and Animal Welfare Committee (No. 2019-A-2-2), and conducted legitimately according to the institutional animal guidelines. During 8 weeks dealt with, the mice' total urinary protein content at the time points of one-week interval was assessed. LN mouse model was established successfully when the urinary protein content was exceeded 1000 mg/l (24 h). The mice were euthanized 8 weeks after the treatment; they were anesthetized with 2% isoflurane (No. 23989, Cayman, USA) and sacrificed immediately by cervical dislocation.

Measurements of urine protein

We collected the 24 h urine of mouse at the time points of one-week interval during 8-week LN model construction. The total urine protein content was measured by CBB method with a commercial kit (No. C035-2-1, Nanjing jiancheng, China) to estimate the renal damaged degree. When the total urine protein content was exceeded 1000 mg/ml (24 h), the mice could be diagnosed as LN.

Table 1. The grouping and operations of mice

Group	Nunber of mice	Intraperitoneal injection	Tail vein injection
NC group	15	saline	saline
Model group	15	pristane	saline
Model+sh-control group	15	pristane	control shRNA interference plasmid
Model+sh-TRAF3 group	15	pristane	TRAF3 shRNA interference plasmid

Renal histology analysis and Masson staining detection

After euthanasia, the mice renal tissues were collected and washed with ice-cold saline. After fixation in 10% buffered formalin (pH 7.4), the renal tissues were embedded in paraffin and cut into 4 μm thick sections. Then, the slices were dewaxed, dehydrated and stained with hematoxylin and eosin (H&E), Masson staining respectively. All the protocols were carried out with the H&E and Masson staining kit (No. G1120 and G1340, Solarbio, China) instructions. Finally, the staining slices were dehydrated, mounted and observed under inverted microscopy. Five renal cortical fields with 200× magnification were randomly selected from each slice. The pathological changes of renal tissues under the microscopy were recorded, and the percentage of pathological fields was calculated.

ELISA

The blood of mice was collected immediately, and centrifuged to obtain the serum. The concentrations of the pro-inflammatory cytokines TNF-α, IL-1β, IL-17a, IFN-γ, IL-10 and IgM, IgG antibody in the serum were detected with ELISA Kit (No. 70-EK2821/2, 70-EK201B/3, 70-EK217/2, 70-EK280HS-24, 70-EK210/4 and 70-EK279-96, Multiscience, China).

Flow cytometry analysis

The lymphocytes were isolated from the mice blood with Ficoll-Hypaque Solution (No. LTS1092P, Tianjin Haoyang, China). Then, the lymphocytes were fixed, permeabilized and labeled with PE conjugated anti-mouse CD40 antibody (No. MA5-17855, Invitrogen, USA) and anti-mouse CD19 antibody (No. MA1-10126, Invitrogen, USA), FITC conjugated anti-mouse CD4 antibody (No. 11-0042-82, Invitrogen, USA). After washed twice, the cells were re-suspended with PBS for flow cytometry analysis. Moreover, the Th1 cells and Th17 cells were also analyzed with flow cytometry analysis. Correspondingly, the $CD4^+$ T cells were intracellularly stained with anti-mouse CD4-FITC (No. 11-0042-82, Invitrogen, USA) and (or) 0.125 μg of anti-mouse IFNγ-PE-Cyanine7 (No. 25-7311-41, Invitrogen, USA) or 0.125 μg of anti-mouse IL-17A-Alexa Fluor 488 (No. 53-7177-81, Invitrogen, USA) after the intracellular fixation and disposition of permeabilization buffer. Finally, the $CD40^+$ B cells, $CD4^+$ T cells sub-types Th1 and Th17 lymphocytes were determined by Fixable Viability Dye (No. 564994, BD Bioscience, USA) in the stated gate.

Western blot

The collected mice renal tissues were digested with collagenase (No. C6079, sigma, USA) and Trypsin (No. 25300054, Gibco, USA) with PMSF (No. P8340, Solarbio, China). The obtained single cells were dissolved in RIPA Lysis Buffer (No. P0013B, Beyotime, China). Afterwards, 25 μg protein of each sample were separated with 8% or 12% sodium dodecylsulfate-polycrylamide gel electrophoresis (SDS-PAGE) gel, and the protein gel was blotted onto polyvinylidene fluoride (PVDF) membranes (No. IPVH15150, Millipore, USA). The membrane was treated with 5% bovine serum albumin (BSA, No. A8020-5, Solarbio, China) in Tris-buffered saline solution, and incubated with primary antibodies against TRAF3 (No. ab217033, Abcam, UK), TRAF2 (No. ab244317, Abcam, UK), NF-κBp52 (No. 4882S, CST, USA), IKKα (No. ab227852, Abcam, UK), ICAM1 (No. ab171123, Abcam, UK) for a whole night. After incubation with second antibody IgG for 2 h, the proteins were detected with staining WesternBreeze™ chemiluminescent kit (No. WB7108, Invitrogen, USA). The protein levels were quantified by the relative concentration to actin protein with the Image Pro Plus Software version 7.0 (Media Cybernetics, USA).

Statistical analysis

Data were shown as mean ± standard error of mean (SEM). Differences between the groups were analyzed with Student's *t* test or one-way ANOVA analysis of variance with SPSS software version 19.0 (SPSS, USA). Besides, $p < 0.05$ was considered statistically significant. All data were presented with GraphPad Prism software version 6.0 (GraphPad Software, USA).

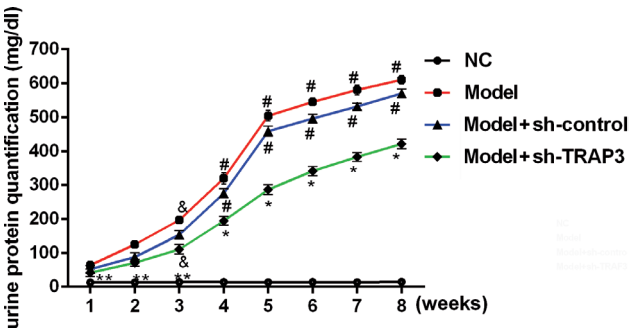

Figure 1. Proteinuria in mice model of lupus nephritis (LN) with TRAF3 knockdown. 24-h urine protein quantification at different time points during 8-week LN model construction. $^{\#}p < 0.05$ *vs.* NC and Model+sh-TRAF3 group from the fourth week. $^{*}p < 0.05$ *vs.* NC group from the fourth week; $^{\&}p < 0.05$ *vs.* Model+sh-TRAF3 at the third week; $^{**}p < 0.01$ *vs.* other groups at the first, second and third weeks. NC, normal control group, the mice i.p. injected with 500 μl vehicle with 0.9% saline; Model, the mice received 500 μl pristine to construct the LN mouse model; Model+sh-control, control shRNA interference plasmid group; Model+sh-TRAP3, TRAF3 shRNA interference plasmid group.

Results

TRAF3 knockdown alleviated the severity of renal damage in LN mouse model

In the LN mouse model, the amount of urinary protein was increased evidently at day 28, but it was reduced in the mice with TRAF3 knockdown by TRAF3 shRNA interference plasmid compared with the LN mouse model and the mice transfected with shRNA control plasmid (Fig. 1, $p < 0.05$). Moreover, the neutrophils were infiltrated in the kidney and the nephritic basement membrane was thickened with partial sclerosis in the LN mouse model, but the pathological lesion in the kidney was alleviated in the mice with TRAF3 knockdown by TRAF3 shRNA interference plasmid (Fig. 2).

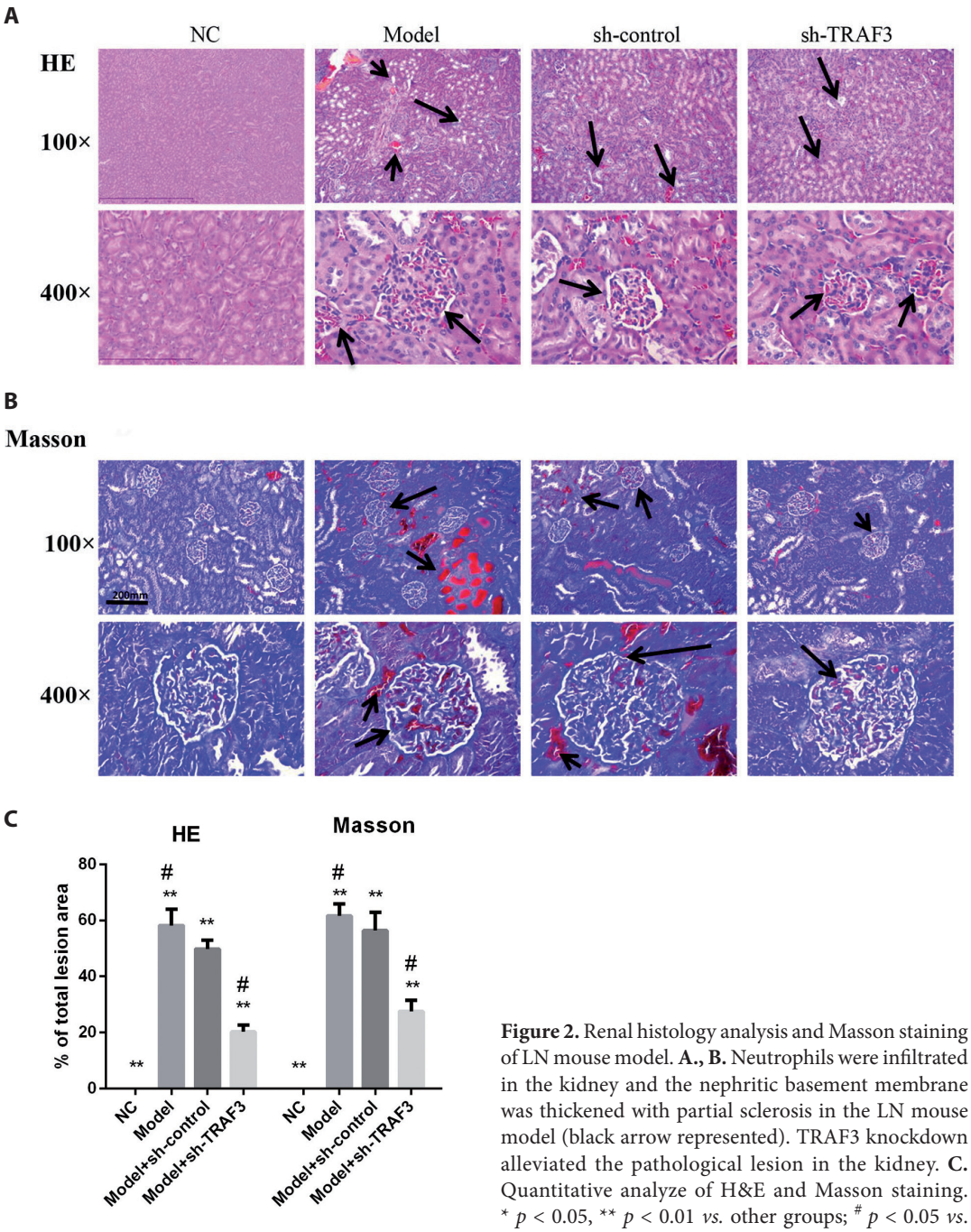

Figure 2. Renal histology analysis and Masson staining of LN mouse model. **A., B.** Neutrophils were infiltrated in the kidney and the nephritic basement membrane was thickened with partial sclerosis in the LN mouse model (black arrow represented). TRAF3 knockdown alleviated the pathological lesion in the kidney. **C.** Quantitative analyze of H&E and Masson staining. * $p < 0.05$, ** $p < 0.01$ *vs.* other groups; # $p < 0.05$ *vs.* Model+sh-TRAF3 group. For abbreviations, see Fig. 1.

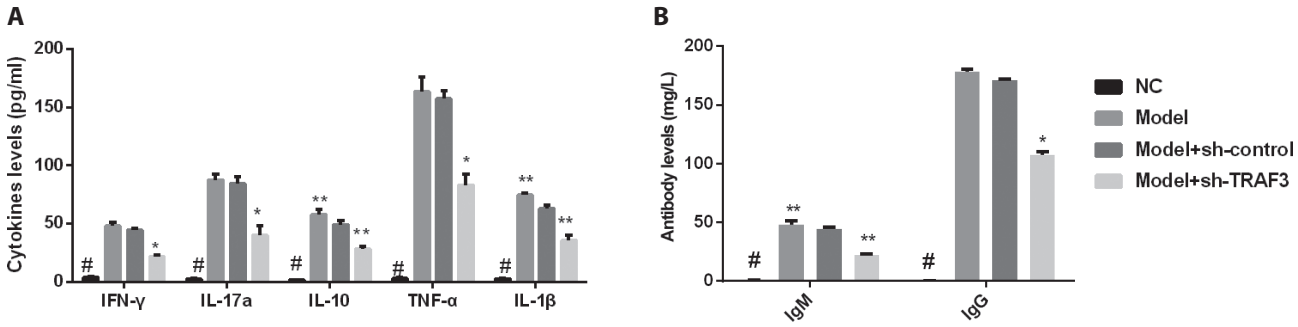

Figure 3. TRAF3 knockdown alleviated the inflammation in LN mouse model. Serum levels of TNF-α, IL-1β, IL-17a, IFN-γ, IL-10 (**A**) and IgM, IgG antibody (**B**) were measured with ELISA. $^{\#}p < 0.05$ *vs.* other groups; $^{**}p < 0.01$ *vs.* Model+sh-TRAF3 group; $^{\&}p < 0.05$ *vs.* Model group and Model+sh-control group. For abbreviations, see Fig. 1.

TRAF3 knockdown alleviated the inflammation in LN mouse model

Inflammation caused by immune dysregulation played important roles in the pathogenesis of LN, so the levels of inflammatory cytokines and IgM, IgG antibody in blood serum were measured with ELISA in our study. Results showed that the pro-inflammatory cytokines, including TNF-α, IL-1β, IL-17a, IFN-γ and IgM, IgG antibody, were enriched in the blood serum of LN mouse model. Whilst, there was little amount of IL-10 inflammatory cytokines secretion in the LN mouse model (Fig. 3), the amount of $CD40^+$ B cells, $CD4^+$ T cells sub-type, Th17 cells were enriched in the blood of LN mouse model (Fig. 4, $p < 0.05$). More importantly, the pro-inflammatory cytokines secretion and the infiltration of $CD40^+$ B cells and Th1, Th17 cells were reduced in the mice with TRAF3 knockdown by TRAF3 shRNA interference plasmid (Figs. 3 and 4).

TRAF3 induced the renal immunologic damage through NF-κB signaling pathway in LN mouse model

The results of Western blot analysis revealed that the proteins TRAF3, TRAF2, NF-κBp52, IKKα, ICAM1 in the kidney were highly expressed in the LN mouse model. Whilst, the expression of these proteins were significantly reduced in the mice with TRAF3 knockdown by TRAF3 shRNA interference plasmid (Fig. 5, $p < 0.05$). Hence, the TRAF3-NF-κB signaling pathway played critical roles in the renal immunologic damage of LN mouse model.

Discussion

Pristane is a kind of membrane active substance, which can be commonly used to induce SLE in mice. It can cause cell death, abnormal secretion of various cytokines, non-specific inflammatory reactions of cell membrane lipids, and formation of various cytoplasmic antigens and autonuclear antigens. In our study, pristane was used to establish LN mouse model, and found the amount of urinary protein was increased, pathological lesion of renal tissue occurred, the pro-inflammatory cytokines TNF-α, IL-1β, IL-17a, IFN-γ and IgM, IgG antibody were enriched in the LN mouse model, showing that the modeling method is feasible and authentic.

In LN mouse model, TRAF3 knockdown was found to significantly alleviate the renal pathological damage, reduce the urine protein content, dramatically reverse the climbing of proinflammatory cytokines and immunoglobulin, such as TNF-α, IL-1β, IL-17a, IFN-γ and IgG, IgM, and reduce the accumulation of $CD40^+$ B cells, $CD4^+$ T cells sub-types Th1, Th17 cells in LN mouse model. Hence, TRAF3 was associated with renal damage and renal immune inflammatory response, indicating that TRAF3 was a key factor to the formation of LN. The study of Ping Xie and colleagues (Xie et al. 2007) revealed that TRAF3 was critical for B cells homeostasis. In our study, the amount of $CD40^+$ B cells were enriched in the mouse model and TRAF3 knockdown reduced the infiltration of $CD40^+$ B cells. So, there was possibility that the interaction between $CD40^+$ B cells ligand and TRAF3 could disrupt self-tolerance to induce humoral immunity, leading to increasing of IgG, IgM production in LN tissues. Besides, the IL-23/IL-17-Th17 axes were found to positively correlate with renal histological injury during the intra-renal inflammation of SLE (Chen et al. 2012). Our results found that the levels of proinflammatory cytokines and Th17 cells were enriched in the LN mouse model, and reduced with TRAF3 knockdown. Therefore, the inflammation induced by proinflammatory cytokines, might also accelerate Th17 expansion in LN.

Researches had identified that upon ligand-receptor binding, TRAF3 could recruit complex TRAF2-cIAPs receptor and compete with NF-κB inducing kinase (NIK) receptor to subsequently activate the noncanonical NF-κB

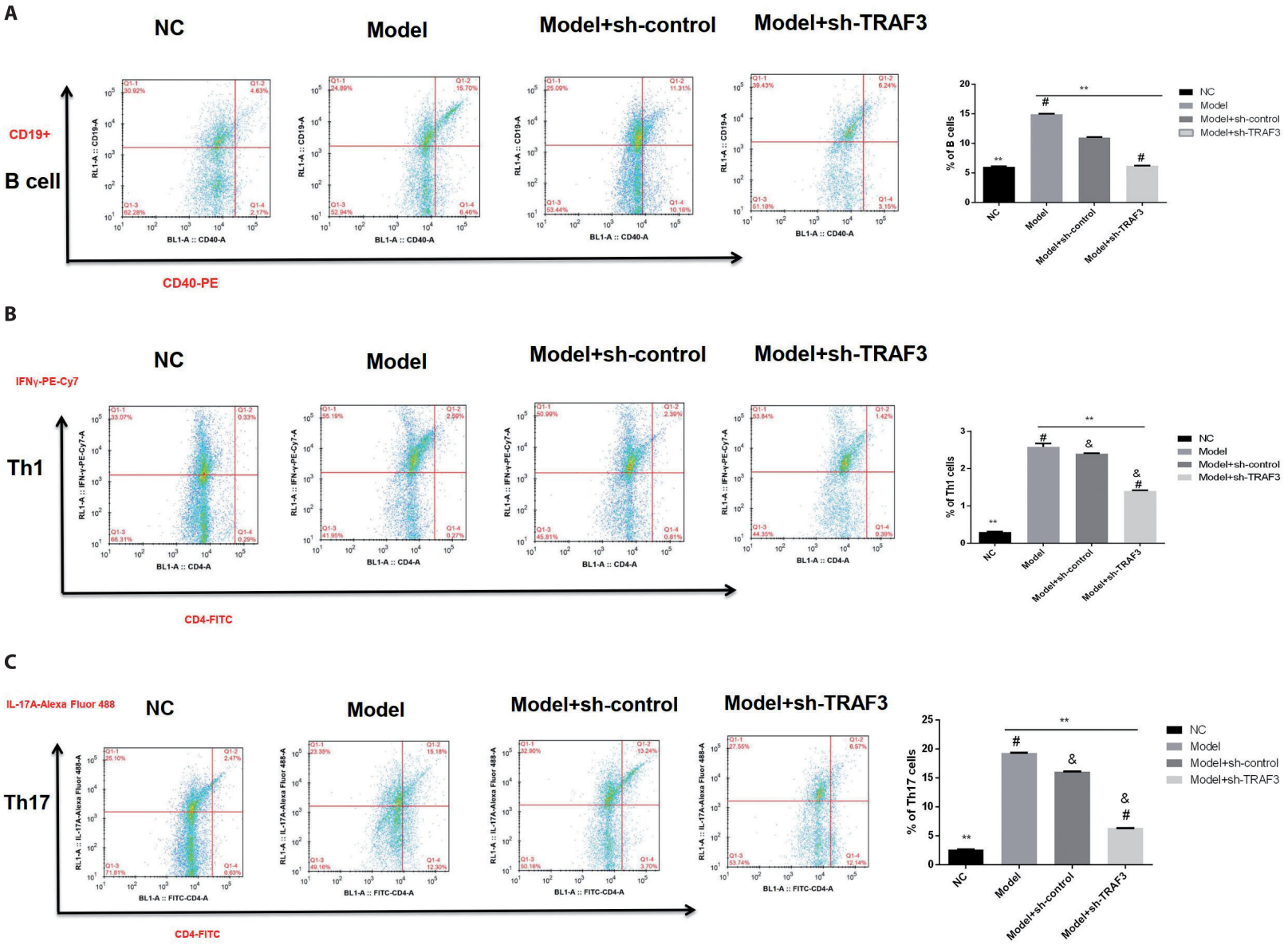

Figure 4. TRAF3 knockdown alleviated the CD40$^+$ B cells (**A**), Th1 (**B**) and Th17 (**C**) cells in LN mouse. The levels of CD40$^+$ B cells, Th1 cells and Th17 cells in mice blood were measured with flow cytometry (left panels) and quantified (right panel). ** $p < 0.01$ *vs.* other groups of CD40$^+$ B cells, Th1 cells and Th17 cells; $^{\#}$ $p < 0.05$ *vs.* Model+sh-TRAF3 group of CD40$^+$ B cells and Th1, Th17 cells; $^{\&}$ $p < 0.05$ *vs.* Model+sh-TRAF3 group of Th1, Th17 cells. For abbreviations, see Fig. 1.

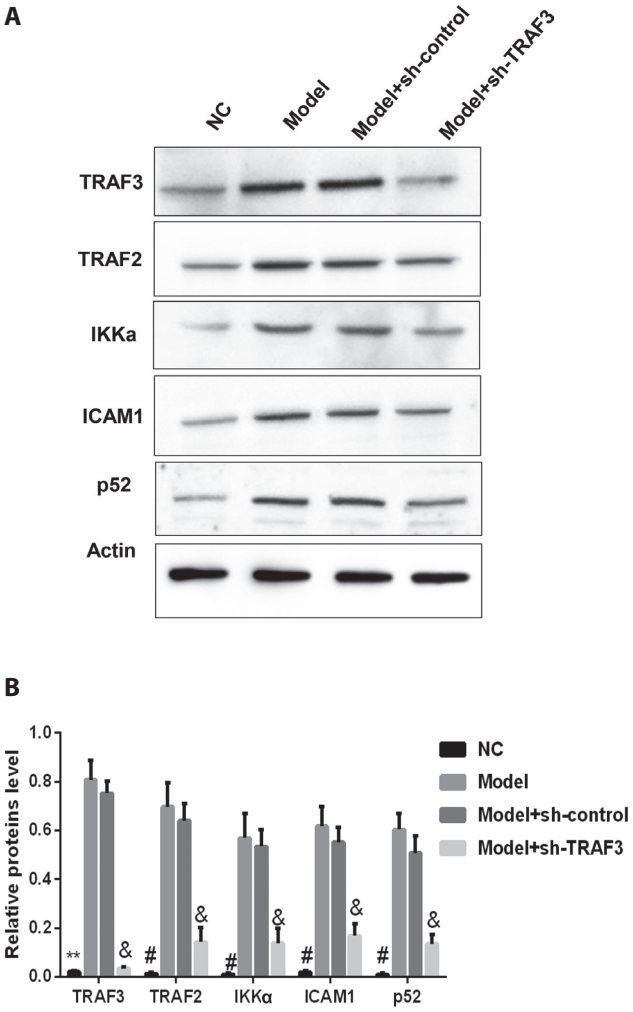

Figure 5. TRAF3-induced LN mouse model through NF-κB signaling pathway. **A.** Expression of TRAF3, TRAF2, NF-κBp52, IKKα, ICAM1 in the kidney were determined with Western blotting. **B.** Quantitative analyze of Western blotting. ** $p < 0.01$ *vs.* Model and Model+sh-control groups; $^{\&}$ $p < 0.05$ *vs.* Model and Model+sh-control groups; $^{\#}$ $p < 0.05$ *vs.* other groups. For abbreviations, see Fig. 1.

signaling pathway (Vallabhapurapu et al. 2008; Razani et al. 2011). Hence, it was speculated that TRAF3-NF-κB signaling was a key pathway in LN disease. To further reveal LN molecular mechanism, the proteins related to NF-κB signaling in kidney were detected, and found that TRAF3 knockdown obviously reduced TRAF2, IKKα, NF-κBp52, ICAM1 proteins' expression, which was consistent with our speculation. Moreover, the infiltration of TNF-α and IL-1β in glomerular endothelial and mesangial cells could activate NF-κB to induce glomerular injury and up-regulated ICAM-1 protein expression in LN (Zheng et al. 2006). In our study, the TNF-α, IL-1β, NF-κBp52, and ICAM1 increased in the LN mouse model, and reduced with TRAF3 knockdown, indicating the relationship between NF-κB signaling and TRAF3 in LN. The activated nucleic transcription factor NF-κB could transcribe multiple pro-inflammatory genes, such as, IL-1, IL-6, IL-12 and TNF-α (Chalmers et al. 2019), which also is corresponding to our results that the expressions of NF-κBp52, IL-1βand TNF-α were increased in the LN mouse model, and reduced with TRAF3 knockdown. Therefore, our results proved from various angles that the TRAF3-NF-κB signaling pathway played a role in LN disease.

However, there are also some limitations and shortcomings. For example, the relationship between TRAF3-NF-κB signaling and LN disease needs to be verified in different strains of mice and animals to obtain more confident conclusions. Moreover, it's necessary to further reveal the association between TRAF3-NF-κB signaling induced renal inflammation and macrophage, DCs induced innate immunity and CD4+ T cell immune response in LN.

In conclusion, this study reveals that TRAF3 plays a key role in LN through the mediation with B cells and Th17 cells as well as cytokines in NF-κB signaling pathway, and studies also are necessary to verify the application of our conclusion, and explore the further molecular mechanisms of LN.

Conflicts of interest. The authors state that there are no conflicts of interest.

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