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MicroRNA-150 Deletion Reduces the Occurrence and Severity of Rheumatoid Arthritis by Inhibiting IL-17

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ABSTRACT

Background: Understanding the effects of epigenetic factors on the pathogenesis of rheumatoid arthritis (RA) is important for the early diagnosis and therapeutic intervention of this disease. MicroRNA-150 (miR-150) exerts an important influence on the development and function of lymphocytes. However, the role of miR-150 in the pathogenesis of RA remains unclear.

Objective: To explore the role of miR-150 in the pathogenesis of RA and the related immune mechanism.

Methods: In this study, we used miR-150 knock-out (miR-150KO) and created animal models of RA. Flow cytometry, immunohistochemistry, and real-time RT-PCR were employed to assess the frequency of T cell subsets and cytokines expression.

Results: Compared to wild-type (WT) mice, the onset of RA was postponed and the incidence of RA was reduced in miR-150KO mice. The expression of IL-4 and IFN-γ significantly increased while the expression of IL-17 decreased significantly in NKT and $CD4^+$ T cells of KO mice compared to that of WT mice after RA induction. In addition, the expression of IL-4 and IFN-γ increased while the expression of IL-17 decreased significantly in the joint tissues of KO mice compared to that of WT mice. Furthermore, the mRNA expression of TNF-α and IL-17 decreased significantly in the synovial fluid cells of KO mice compared to that of the WT mice after RA induction.

Conclusion: MiR-150 deficiency decreases the expression of IL-17 in T cells and joint tissues, and alleviates the occurrence and progression of RA in mice.

Keywords: IL-17, MicroRNA-150, Rheumatoid Arthritis

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INTRODUCTION

MicroRNAs (miRNAs) are a class of non-coding single-stranded RNA molecules with a length of about 22 nucleotides. Although in general, miRNAs inhibit the expression of their target genes by direct mRNA cleavage or transcriptional repression, they may positively regulate gene expression under certain conditions. One gene can be targeted by several different miRNAs, and one miRNA can also have several target genes. Therefore, miRNAs play an important role in many biological processes, such as cell proliferation, differentiation and immune functions (1).

MiR-150 is mainly expressed in lymphoid organs and plays a key regulatory role in lymphocyte development and differentiation. For example, the development and differentiation of both B cells and $CD8^+$ T cells are controlled by miR-150 through the transcription factor c-myb (2, 3). In addition, our previous studies demonstrated that miR-150 regulated the development of natural killer T (NKT) cells in the thymus through c-myb, and miR-150 deletion increased IFN-γ expression of peripheral NKT cells as well (4). Besides, miR-150 repressed inflammation by decreasing the expression of TNF-α and IL-6, but increasing the expression of IL-10 in lipopolysaccharide (LPS)-induced inflammatory response (5, 6). Additionally, miR-150 expression closely associated with pathologic changes of autoimmune diseases (7-9).

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by erosive arthritis. The pathological basis of RA is synovial inflammation and structural damage caused by infiltration of inflammatory cells and pro-inflammatory cytokines. It is well known that TNF-α extensively participates in synovial inflammation and structural damage of RA (10). Moreover, IL-17 can further strengthen the expression of pro-inflammatory cytokines (TNFα, IL-6 and IL1β) and matrix metalloproteinases in RA (11).

The role of miR-150 in RA is still undetermined. Chen Z, et al. reported that miR-150 expression decreased in the serum of RA patients compared with that of osteoarthritis patients, and over-expression of exosome derived miR-150 alleviates RA (12). While other studies showed that miR-150 expression increased in the peripheral blood mononuclear cells (PBMCs) of RA patients compared with that of healthy persons, which was also positively associated with more severe inflammation and destruction of joints (13). In any case, the above reports indicated that miR-150 played a role in the occurrence or progression of RA disease. Thus, by using miR-150 knock-out (KO) mice and collagen-induced arthritis mouse model, we further explored the effect of miR-150 on RA and its preliminary immunological mechanisms in this study.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6 mice and microRNA-150 knock-out (miR-150KO) mice with C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, USA). All the mice were fed in a specific pathogen-free animal room with room temperature of 20–25°C and room humidity of 40–60%. Male (10-16 weeks old) WT and KO mice were used in this study. Mouse disposal and experimental procedures were in accordance with the stipulations of Laboratory Animal Ethics Committee of North China University of Science and Technology.

Genotyping

Mouse tail DNA and the following PCR primers were used to genotype the WT and the KO mice. Forward: 5'-CAAGGACAGGAACCCTTCAGCA-3', reverse: 5'-CCATGATG CCTGGAAGACA TTTC-3'. The WT and the KO mice produce DNA fragment of 866bp and 262bp respectively after PCR amplification.

Induction and Clinical Evaluation of RA

The mice were divided randomly into four groups (n=10 per group) including the wild-type control (WT-control), the WT-RA, the miR-150 KO control (KO control) and the miR-150 KO RA (KO RA). All the mice were co-housed in individual cages (maximum n=5 per cage), and they had free access to food and water under a twelve-hour light/dark cycle during experiment. The induction of RA was performed according to the protocol recommended by KM. Pietrosimone1 et al. with some modifications (14). Type II chicken collagen (Chondrex, USA) was first dissolved in 0.01 M acetic acid overnight at 4°C and then emulsified with the same amount of Freund's complete adjuvant (FCA, Chondrex). The mice in the WT-RA and KO-RA group were injected intradermally with 100 μL chicken collagen emulsion (100 mg) at the base of tail. The mice in the WT-control and the KO-control group were treated with the same amount of acetic acid and FCA solution. 21 days after the initial immunization, the mice in WT-RA and KO-RA group were administered 100 mg collagen emulsion as a booster injection, while the mice in the WT-control and the KO-control group were still treated with the same amount of acetic acid and FCA solution. The thickness of mouse's paw in each group was measured with caliper to monitor arthritis, and the severity of arthritis was evaluated based on arthritis index (AI) on a scale of 0–3 (0=normal, 1=slight joint swelling and/or erythema, 2=obvious swelling and/or erythema for more than one joints, 3=pronounced swollen and ankylosis of joints and paws). A maximum of 12 points can be scored by adding the AI score of all four mouse limbs.

Flow Cytometry Analysis

Fresh splenic and inguinal lymph node cells were separated by mincing and dissolved in PBS buffer. The cell suspensions were filtered with a 40-μm cell strainer and centrifuged at 350 ×g for 5 min at 4□. Cell pellets were then suspended in a 2ml staining buffer [PBS with 2% fetal calf serum (FCS)] and incubated with the Fc Block reagent (clone 2.4G2) for 10 min at 4□.The following fluorochrome-labeled antibodies were adopted for cell surface staining: anti-mouse-CD4-PerCP-Cy5.5 (Biolegend, 100433), anti-mouse-CD8-FITC(BD,551162), anti-mouse-TCR-β-FITC (eBiosciences, MA1-70045), CD1d-α-Galcer tetramer-PE (TET, Kirin Corporation of Japan). The cell suspension (100 μl) was incubated with the mAbs or TET at 4□ for 30 min, washed with PBS buffer, and then analyzed with a BD FacScanto II or Beckman flow cytometer. The data were analyzed by CELLQuest Pro or FlowJo software.

Intracellular Cytokine Staining

Splenic cells from the WT-control, the KO-control, the WT-RA and the KO-RA mice were suspended in RPMI1640 cell culture medium in the presence of PMA (50 ng/ml) and ionomycin (1 uM) for 1 h at 37□. Golgistop was added at a final concentration of 3 uM and continued to culture for 3 h. The cells were collected and first performed by cell surface staining. Then, the cells were fixed and permeated with a Cytofix/Cytoperm kit (BD Biosciences, 554722). After washing with PBS, the cells were intracellularly stained with anti-mouse-IFN-γ-APC (eBioscience, E00862-16320), anti-mouse-IL-4-APC (Biolegend, 504105) and anti-mouse-IL-17A-APC (eBioscience, 17-7177-81) for 30 min at 4□, washed with PBS buffer, and then analyzed with a BD FacScanto II or Beckman flow cytometer. The data were analyzed by CELLQuest Pro or FlowJo software.

Histology Examination (H&E) and IHC

Ankle joint tissues from the WT-control, the KO-control, the WT-RA and the KO-RA mice were first decalcified with EDTA decalcification agent and then stained with H&E. The dyeing procedure was as follows: hematoxylin soaked for 10 min, water flushed for 10 min, alcohol hydrochloride decolored for 10 s, eosin stained for 1min, washed

twice, dehydrated with alcohol from low to high concentration and then transparent processed in xylene for 10 min; after the xylene volatilization, the slices were sealed with neutral gum and observed with an optical microscope. 200 cells in each tissue slice were observed under a 400-fold microscope. The degree of inflammatory cell infiltration in ankle joint tissues was calculated as follows: percentage of inflammatory cell=T/200*% (T: total number of inflammatory cells observed). The observation and counting were repeated for three times.

IHC was performed using a streptavidin peroxidase kit (Haling biological technology company, China) according to the operations manual. Tissue slides of ankle joint of mice in each group were first treated by normal goat serum to block nonspecific binding, then incubated with primary antibodies for mouse IFN-γ, IL-4 and IL-17 (Santa Cruz Biotechnology, USA) overnight at 4□. After washing, the slides were incubated with biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase for 20 min at 37□. Diaminobenzene and hematoxylin were used respectively for color development and counterstaining. Immunohistochemical scores (IHS) were determined as follows: IHS=A×B. A represents grading of positive cells: 0=0~1%, 1=1%~10%, 2=10%~50%, 3=50%~80%, 4=80%~100%. B represents grading of color intensity of positive cell: 0=negative, 1=weak positive, 2=positive, 3=strong positive.

Real-time RT-PCR

Synovial fluid (SF) cells of the joint cavity from the WT-control, the KO-control, the WT-RA and the KO-RA mice were rinsed by PBS and collected in a 15ml centrifuge tube. Total RNA was extracted by TRIzol Kit (Takara, Japan) and reverse transcribed with Oligo dT according to the kit instructions. DyNAmo SYBR Green qPCR kit (Finnzymes, Finland) was used to perform PCR in a Real-time Fluorescent Quantitative PCR System (Roche, Switzerland). The thermal conditions were as follows: 95□ for 5 min, 40 cycles of 95□ for 20 s, 60□ for 30 s, and 72□ for 15 s. The expression of TNF-α, IL-17a, IFN-γ and IL-4 was calculated by a relative quantification method and hypoxanthine phosphoribosyl transferase (HPRT) was used as an endogenous reference. The following primers were used as: HPRT forward, 5'-AGTACAGCCCCAAAATGGTT AAG-3', HPRT reverse, 5'-CTTA GGCTTTGTATTTGGCTTTTC-3'. IFN-γ forward, 5'-GAACTGGCAAAAGG ATGGTGA-3', IFN-γ reverse, 5'-TGTGGG TTGTTGAC CTCAAAC-3'. TNF-α forward, 5'-GAGTGACAAGCCTGTAGCC-3', TNF-α reverse, 5'-CTCCTGGTATGAG ATAGCAAA-3'. IL-17a forward, 5'-CTC AGACTACCTCA ACCGTTCC-3', IL-17a reverse, 5'-ATGTGGTGGTCCAGCTTTCC -3'. IL-4 forward, 5'-TTGTCATCCTGCT CTTCTTTCTC-3', IL-4 reverse, 5'-CAGGA AGTCTTTCA GTGATGTGG-3'.

Statistical Analysis

SPSS17 software was employed for the data processing and statistical analysis. One-way ANOVA and Student's t-test were used to analyze the statistical difference of multiple or two groups of samples respectively. The data were presented as the mean±SD, and $p<0.05$ was considered statistically different.

RESULTS

MiR-150 Deletion Ameliorates RA

Genotyping of the WT and the KO mice showed successful deletion of *miR-150* gene in the KO mice (Fig. 1A). Fig. 1B showed that no mice in the WT-control and the KO-control group developed RA. Only the mice in the KO-RA and the WT-RA group developed RA after boost immunization at day 21. Furthermore, the onset of RA was earlier and the average disease score of RA was higher in the WT

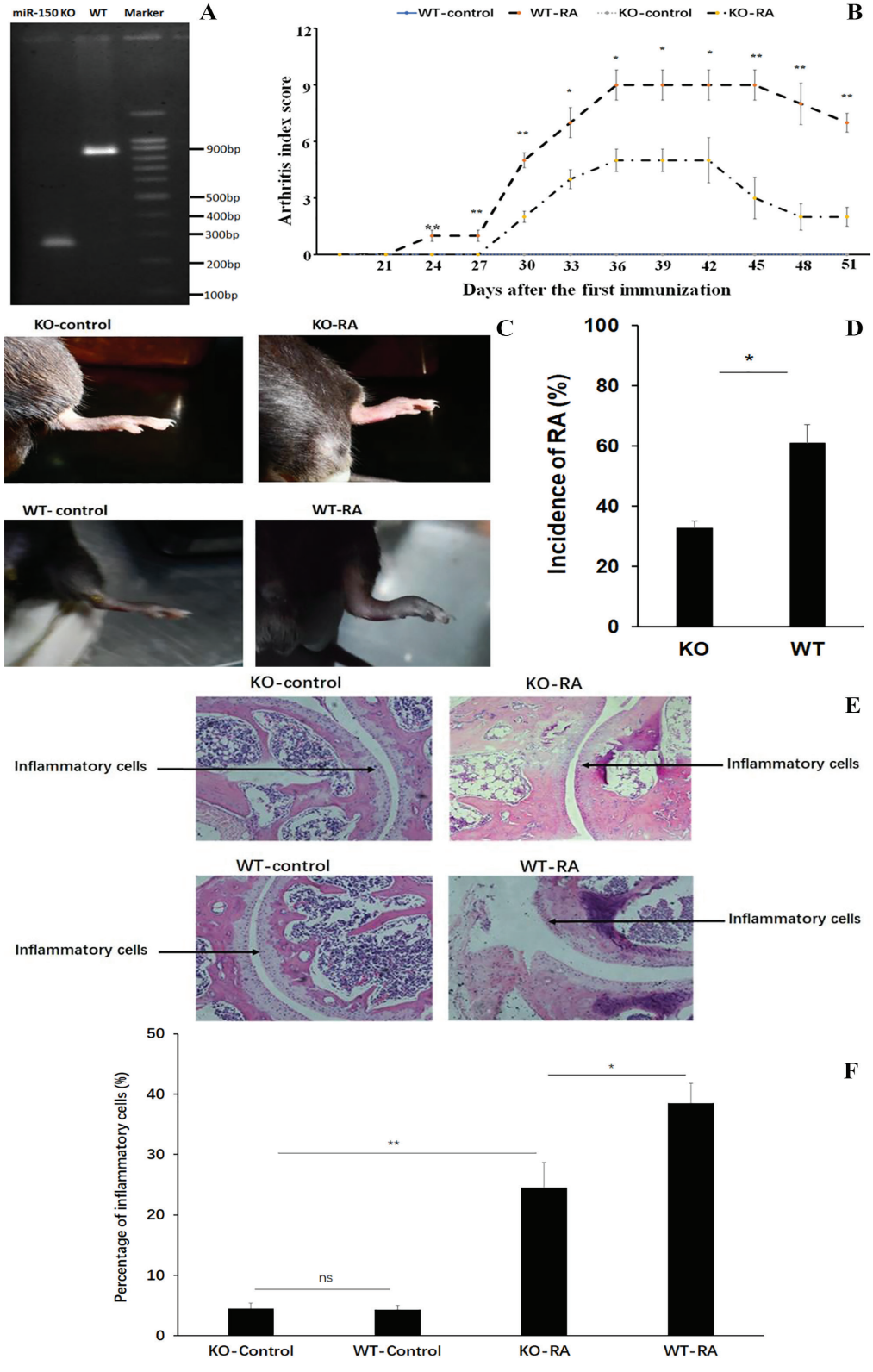

Fig. 1. Deletion of miR-150 ameliorates rheumatoid arthritis. (A) Representative genotyping of wild type (WT) and miR-150 knock-out (KO, KO) mice by PCR. (B) Differences in onset and average disease score of rheumatoid arthritis in mice of the WT-control, the KO-control, the WT-RA and the KO-RA group (C) Representative hind-foot of mice in different groups of mice at day 51 after RA induction. (D) Differences in incidence of rheumatoid arthritis in mice of the WT-RA and the KO-RA groups at day 51 after RA induction. (E) Representative H&E staining of ankle joints in different group of mice at day 51 after RA induction. (F) Statistical analysis of inflammatory cell infiltration to the ankle joint in different groups of mice. *p<0.05. **p<0.01.

mice than that of the KO mice ($p<0.01$). When observed at day 51 after RA induction, the hind-foot swelling of WT mice was more obvious than that of the KO mice (Fig. 1C). Accordingly, the incidence of RA increased significantly in mice of WT-RA group compared with that of the KO-RA group ($p<0.05$, Fig. 1D). There was no structural damage and little inflammatory cell infiltration in ankle joint of mice of the WT-control and the KO-control group. However, the damaged structure and infiltration of inflammatory cells were observed in ankle joint of mice of the WT-RA and the KO-RA group. Furthermore, the percentage of inflammatory cells infiltrated in ankle joint of mice of the WT-RA group was significantly higher than that of the KO-RA group ($p<0.05$, Figs. 1E and 1F). The above results showed that miR-150 deletion inhibited the occurrence and development of RA in mice.

MiR-150 Does not Affect the Ratio of T Cell Subsets in Lymphoid Organs of RA Mice

Because changes of T cell subsets closely related to the occurrence and development of RA. $CD4^+$, $CD8^+$ T cells and NKT cells in the spleen (SP) and lymph nodes (LNs) of mice were detected by flow cytometry at day 51 after RA induction. Unexpectedly, no significant difference between $CD4^+$, $CD8^+$T cells and NKT cells was found in the lymphoid organs of the WT-Control, the KO-Control, the WT-RA and the KO-RA mice (Figs. 2A-2E). These results suggested that the effect of miR-150 on RA had little relationship with the ratio changes of T cell subsets.

MiR-150 Regulates IL-4, IFN-γ and IL-17 Expression in T Cell Subsets of RA Mice

Next, the expression of IL-4, IFN-γ and IL-17 in NKT and $CD4^+$ T cells was detected by intracellular staining. IFN-γ increased significantly in NKT cells of the WT-RA ($p<0.05$) and the KO-RA ($p<0.01$) mice compared with that of the WT-control and the KO-control mice. Furthermore, IFN-γ increased significantly in NKT cells of KO-RA mice compared with that of WT-RA mice ($p<0.01$). In fact, we saw a higher expression of IFN-γ in NKT cells of KO-control mice than that of WT-control mice ($p<0.05$, Figs. 3A and 3B). IL-4 increased significantly in NKT cells of KO-RA mice compared with that of WT-RA, WT-control and KO-control mice ($p<0.05$). However, IL-17 decreased significantly in NKT cells of KO-RA mice compared with that of WT-RA mice ($p<0.05$), though it increased significantly in WT-RA and KO-RA mice compared with that of WT-control and KO-control mice ($p<0.01$, Figs. 3A and 3B).

IFN-γ, IL-4 and IL-17 expression increased significantly in CD4+T cells of the WT-RA and the KO-RA mice compared with that of WT-control and KO-control mice ($p<0.01$, Figs. 3C and 3D). In addition, IFN-γ ($p<0.01$) and IL-4 ($p<0.05$) increased significantly in $CD4^+$ T cells of the KO-RA mice compared with that of the WT-RA ones. However, IL-17 decreased significantly in $CD4^+$ T cells of the KO-RA mice compared with that of the WT-RA mice ($p<0.05$, Figs. 3C and 3D). The above results indicated that miR-150 regulated the function of T cell subsets in RA mice.

MiR-150 Regulates IL-4, IFN-γ and IL-17 Expression in Ankle Joints of RA Mice

Production of inflammatory cytokines in joint tissues is directly related to the progression of RA. IFN-γ, IL-4 and IL-17 in joint tissues of the KO and the WT mice were evaluated by IHC after RA induction. As shown in Fig. 4, there was little expression of IFN-γ, IL-4 and IL-17 in WT-control and KO-control mice, although, they were expressed in both the KO-RA and the WT-RA mice. In addition, IFN-γ ($p<0.01$) and IL-4 ($p<0.05$) increased significantly in the joint tissues of the KO-RA mice compared with that of the WT-RA mice (Figs. 4A, 4B, 4C and 4D), IL-17 instead decreased significantly in the joint tissues of the KO-RA mice compared with that of the WT-RA mice ($p<0.05$, Figs. 4E and 4F).

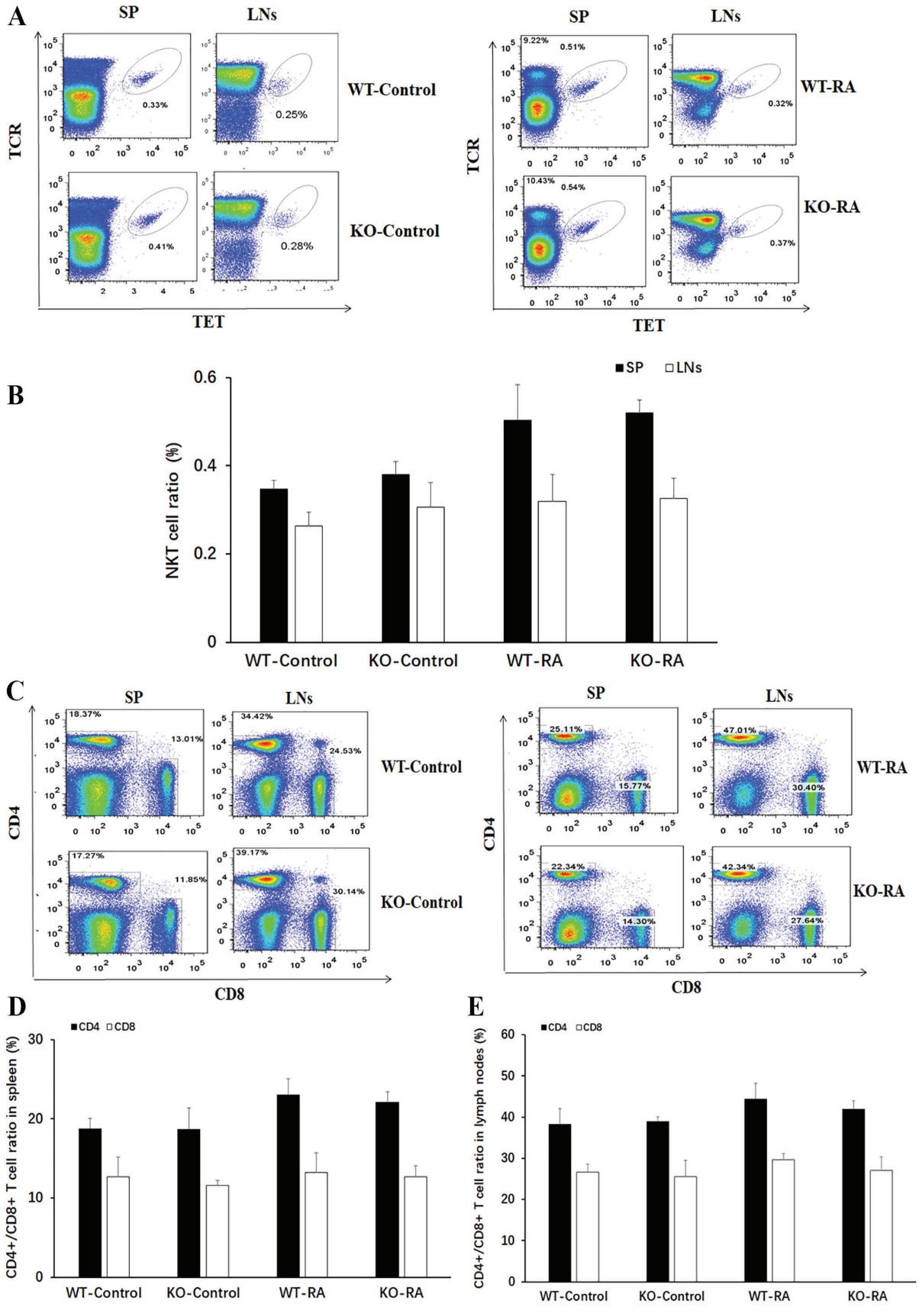

Fig. 2. MiR-150 does not affect the ratio of T cell subsets in lymphoid organs of RA mic. Representative gating and statistical analysis of TCR+ Tet+ NKT (A and B), CD4+ and CD8+ T cells (C, D and E) from SP and LNs of WT and miR-150 KO mice at day 51 after RA induction.

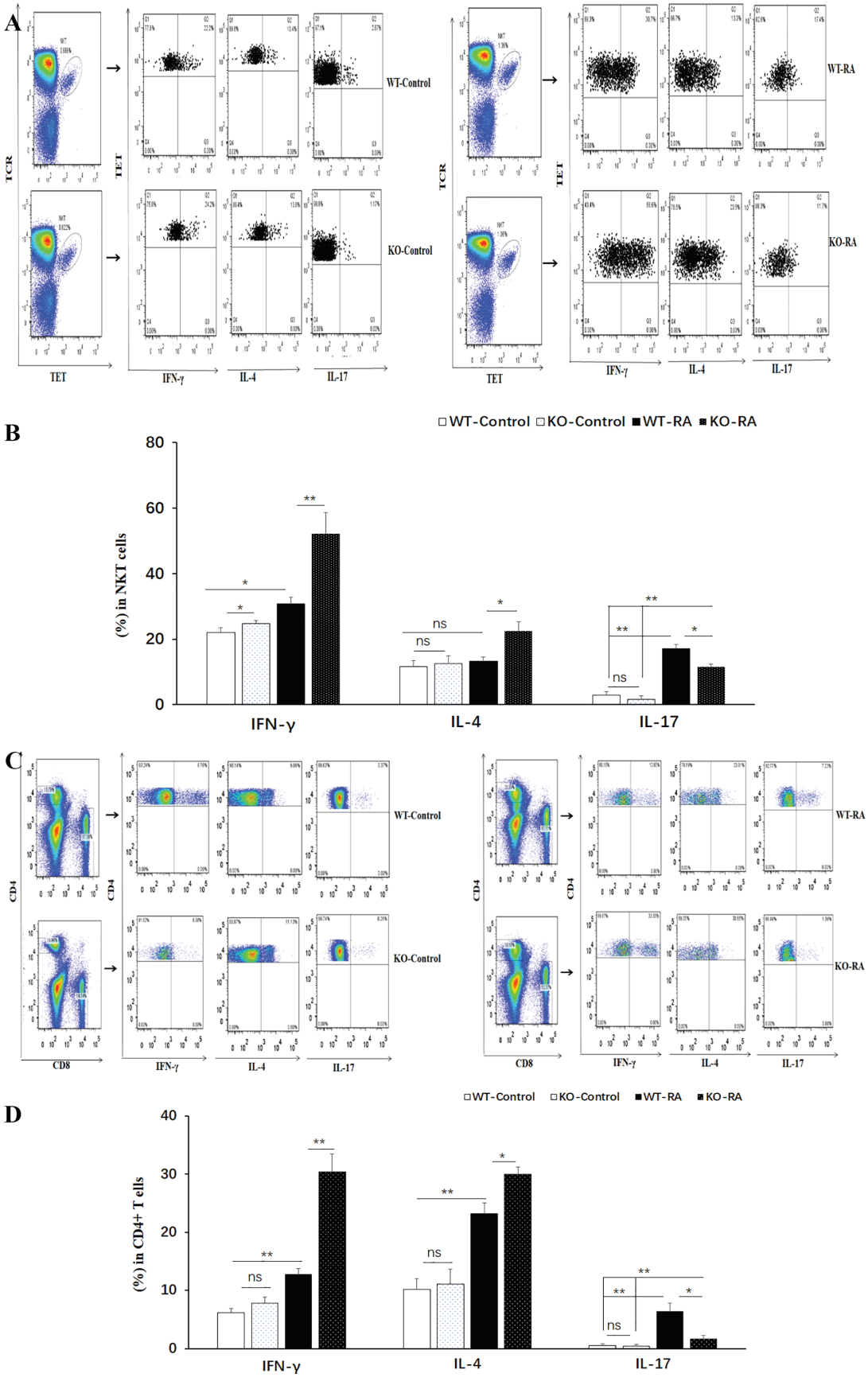

Fig. 3. Effects of miR-150 deletion on IL-4, IFN-γ and IL-17 expression in NKT and CD4+T cells of RA mice. Representative gating and statistical analysis of IL-4, IFN-γ and IL-17 positive cells in NKT (A and B) and CD4+ T cells (C and D) of WT and miR-150 KO mice at day 51 after RA induction. *p<0.05, **p<0.01.

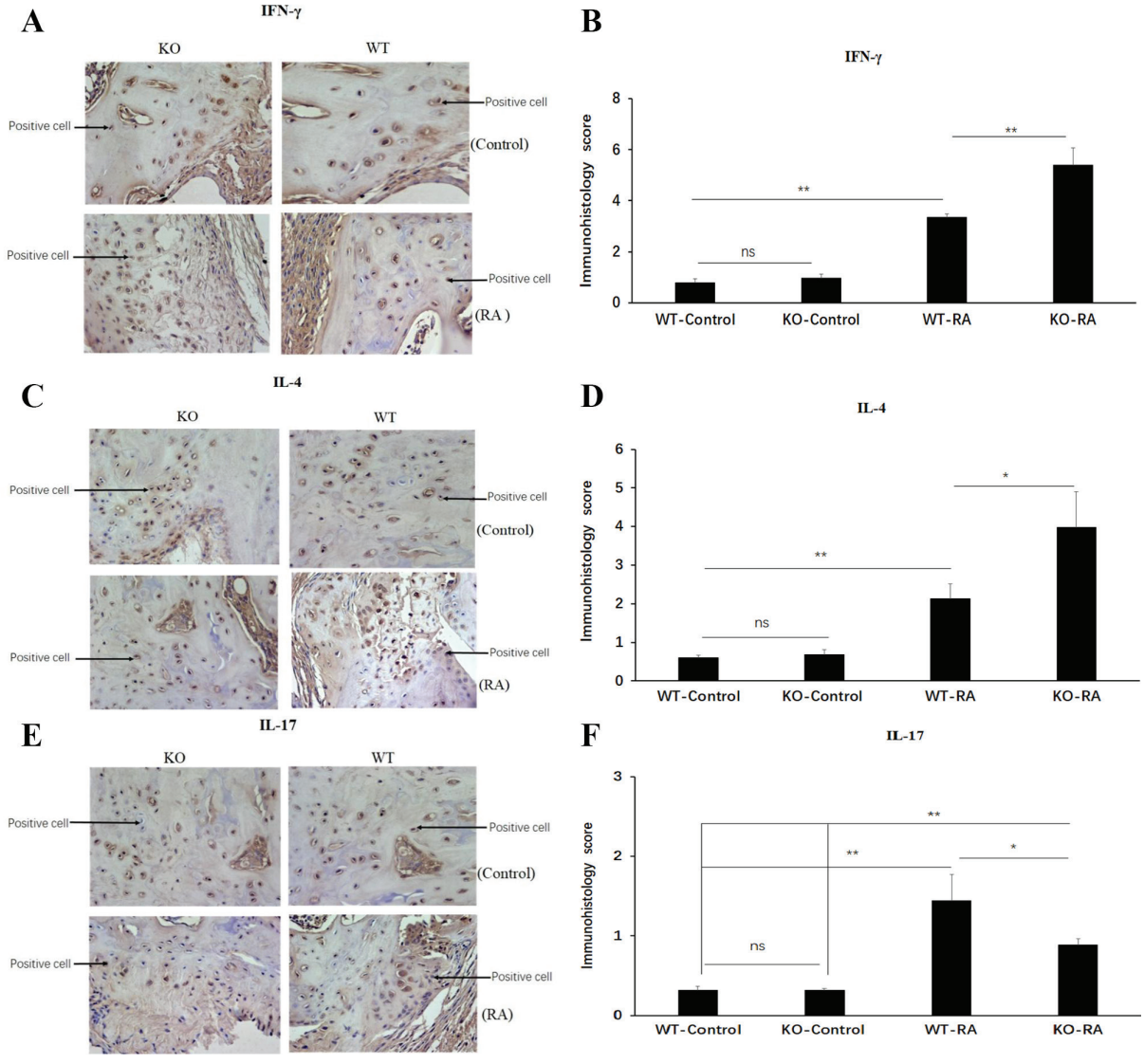

Fig. 4. MiR-150 regulates IL-4, IFN-γ and IL-17 expression in ankle joints of RA mice. IHC staining and statistical analysis of IFN-γ (A and B), IL-4 (C and D), and IL-17 (E and F) expression in ankle joint tissues of WT and miR-150 KO mice at day 51 after RA induction. *p<0.05, **p<0.01.

MiR-150 Regulates TNF-α, IL-4, IFN-γ and IL-17 Expression in SF Cells of RA Mice

Real time RT-PCR was used to assess the expression TNF-α, IFN-γ, IL-4 and IL-17 in SF cells of mice. As shown in Fig 5, TNF-α, IL-4, IFN-γ and IL-17 increased significantly in SF cells of the KO-RA and the WT-RA mice compared with that of the KO-control and the WT-control mice (p<0.01). In addition, TNF-α (p<0.01) and IL-17 (p<0.05) increased significantly in SF cells of the WT-RA mice compared with that of the KO-RA mice. Interestingly, the expression of IFN-γ and IL-4 in the SF cells of the WT-RA mice also showed an increasing trend compared with that of the KO-RA mice, although they were not significantly different. These results indicated miR-150 positively regulated expression of pro-inflammatory cytokines in SF cells of RA mice.

DISCUSSION

RA is an autoimmune disease characterized by chronic systemic and joint inflammation. So far, the etiology of RA has not been completely elucidated. MiRNAs are widespread in eukaryotes and regulate the expression of other genes through the inhibition of

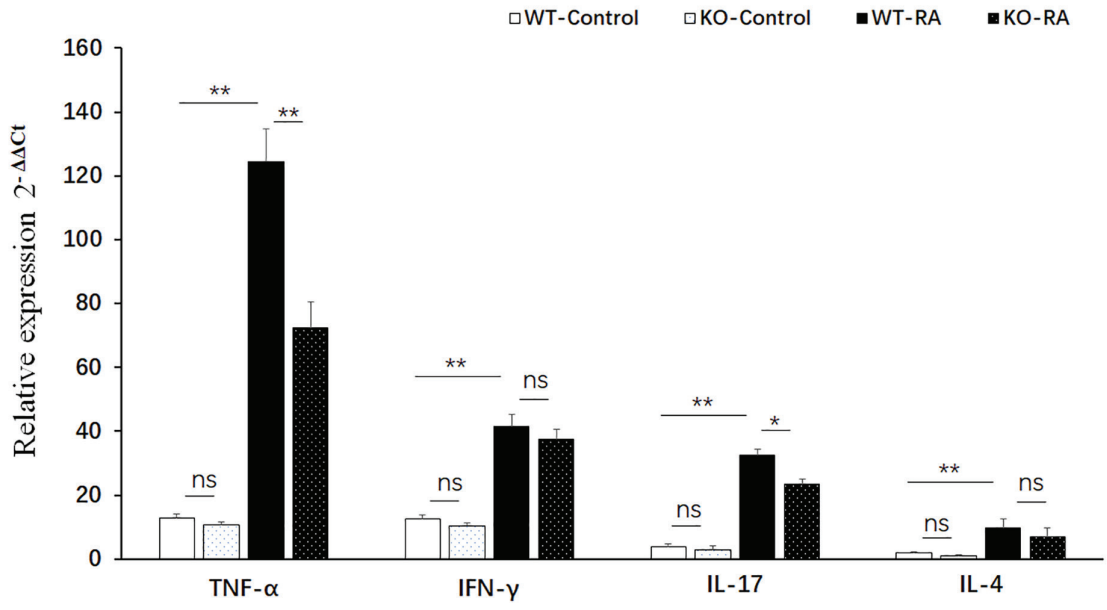

Fig. 5. MiR-150 regulates TNF-α, IL-4, IFN-γ and IL-17 expression in SF cells of RA mice. Real time RT-PCR analysis of TNF-α, IFN-γ, IL-4 and IL-17 expression in SF cells of the KO and the WT mice at day 51 after RA induction. *p<0.05, **p<0.01.

transcription and translation. Recent studies, however, have demonstrated that miRNAs were also involved in RA pathogenesis (15). In this study, we investigated the role of miR-150 in RA by using miR-150 knock-out (KO) mice and collagen-induced arthritis mouse model (CIA). We found that miR-150 deletion resulted in milder symptoms of RA as indicated by lower clinical scores and joint tissue injury after RA induction (Fig. 1). Thus, our data supported that miR-150 may exacerbate disease progression in RA.

Recent studies showed that the abnormal changes of T cell subsets, such as $CD4^+$, $CD8^+$ T cells and NKT cells are involved in RA occurrence and progression (16-19). However, we found out the comparable number of $CD4^+$, $CD8^+$ T cells and NKT cells in the lymphoid organs of the WT and the KO mice after RA induction (Fig. 2). These results indicated that functional changes of T cell subsets may be involved in RA progression in the KO mice.

NKT cells are characterized by co-expressing TCR and NK cell receptors, and they can rapidly secrete massive amounts of cytokines, such as IL-4, IFN-γ and IL-17 after activation (20). NKT cell-deficient mice showed a remission of RA symptoms (21). In addition, convincing evidence showed $CD4^+$ T cells were involved in chronic autoimmune diseases through abnormal expression of Th1/Th2/Th17-associate cytokines (22). We found out that miR-150 deletion significantly increased the expression of IL-4 and IFN-γ, but decreased the expression of IL-17 in both T cell subsets and ankle joint tissues of mice after RA induction (Figs. 3, 4). It is well known that IL-17 plays a critical role in both the initiation and bone destruction phase of autoimmune arthritis (23-25). However, the effects of IL-4 and IFN-γ on RA pathogenesis were controversial. Some studies reported that increased expression of IL-4 and IFN-γ was associated with accelerated RA progression (26-28). While other studies showed a protective role of IL-4 and IFN-γ in RA and CIA (29-32). Our data in this study suggested that miR-150 deletion led to a disturbance of Th1/Th2/Th17 cytokine expression in the immune organs and joint tissues, which may synergistically mitigate the development and pathogenesis of RA in mice.

In RA, the cellular origin of TNF-α mainly comes from synovial $CD4^+$ T cells and macrophages (33). In addition, miR-150 deletion decreased IL-17 and TNF-α

expression, while miR-150 overexpression increased TNF-α expression in CD4+ T cells of experimental autoimmune encephalomyelitis (EAE) mice (8, 34). Consistent with these reports, we found out that TNF-α and IL-17 expression in SF cells of the KO mice was significantly decreased compared with that of the WT mice (Fig. 5). In addition, IFN-γ and IL-4 expression also showed a decreasing trend in SF cells of the KO mice compared with that of the WT mice after RA induction (Fig. 5). Although the exact mechanism is unknown at present, it is probably because miR-150 deletion leads to an immune tolerance state in the joint cavity by inhibiting cytokine release and synovial cell activation after RA challenge. Moreover, IL-17 may play a dominant role in RA disease progression mediated by miR-150.

CONCLUSION

In summary, this study demonstrated that miR-150 increased inflammatory responses and disease progression by disturbing Th1/Th2/Th17 cytokine production in RA mice, bringing new insights into the diagnosis and treatment of RA. The mechanisms of miR-150 in regulating the expression of Th1/Th2/Th17 cytokines in T cell subsets and SF cells of RA mice are not known at present. Thus, more detailed studies focusing on the relationship of miR-150 and immune molecules in RA should be conducted in the future to validate the present findings.

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AUTHORS' CONTRIBUTION

QZ designed the study; AZ and QZ performed all the experiments and wrote the manuscript. AZ collected and analyzed the data. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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