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Research Article

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Research Article

TAGAP activates Th17 cell differentiation by promoting RhoA and NLRP3 to accelerate rheumatoid arthritis development

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that can give rise to joint swelling and inflammation, potentially affecting the entire body, closely linked to the state of T cells. The T-cell activation Rho GTPase activating protein (TAGAP) is associated with many autoimmune diseases including RA and is directly linked to the differentiation of Th17 cells. The present study intends to investigate the influence of TAGAP on the RA progression and its mechanism to empower new treatments for RA. A collagen-induced-arthritis (CIA) rat model was constructed, as well as the extraction of $CD4^+$ T cells. RT-qPCR, H&E staining and safranin O/fast green staining revealed that TAGAP interference reduced TAGAP production in the ankle joint of CIA rats, and joint inflammation and swelling were alleviated, which reveals that TAGAP interference reduces synovial inflammation and cartilage erosion in the rat ankle joint. Expression of inflammatory factors (TNF-α, IL-1β, and IL-17) revealed that TAGAP interference suppressed the inflammatory response. Expression of pro-inflammatory cytokines, matrix-degrading enzymes, and anti-inflammatory cytokines at the mRNA level was detected by RT-qPCR and revealed that TAGAP interference contributed to the remission of RA. Mechanistically, TAGAP interference caused a significant decrease in the levels of RhoA and NLRP3. Assessment of Th17/Treg levels by flow cytometry revealed that TAGAP promotes Th17 cells differentiation and inhibits Treg cells differentiation *in vitro* and *in vivo*. In conclusion, TAGAP interference may decrease the differentiation of Th17 cells by suppressing the expression of RhoA and NLRP3 to slow down the RA progression.

Keywords: TAGAP, gene interference, rheumatoid arthritis, Th17 cell, NLRP3 inflammasome

Abbreviations: CFA: complete Freund's adjuvant; CIA: collagen-induced-arthritis; EDTA: ethylenediaminetetraacetic acid; GAPDH: anti-glyceraldehyde phosphate phosphate dehydrogenase; MACS: magnetic-activated cell sorting; NC: normal control; PBS: phosphate-buffered saline; PMA: phorbol 12-myristate 13-acetate; RA: rheumatoid arthritis; SPF: specific pathogen free; TAGAP: T-cell activation Rho GTPase-activating protein; Th17: T helper type 17; TNF: tumor necrosis factor.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disease that affects the quality of life in mild cases and causes disability in severe cases. It is mainly characterized by synovial hyperplasia, vascular opacification and destruction of bone and cartilage in synovial joints, resulting in joint pain, swelling and loss of function, which significantly affects the quality of life of patients [1, 2]. The cause of RA has not been fully revealed, but studies have confirmed that dysregulation of the immune system plays an important role in the development of the disease. The joint inflammation manifested by RA is usually caused by infiltrating macrophages and various pro-inflammatory factors secreted by T and B cells in synovial fluid and tissues that promote the inflammatory cascade, such as interleukins IL-1β, IL-6, IL-8, TNF-α, chemokines, and interferon family [3]. Ahmad *et al.* have discovered that protein tyrosine kinases [4], histamine 4 receptor [5], CXCR3 [6], and other factors contribute to the progression of inflammation and immune responses in RA, exacerbating the severity of the condition. Conversely, the inhibitors or antagonists of these factors have the potential to alleviate RA symptoms. As such, how to regulate the imbalance of the immune system and reduce the secretion of pro-inflammatory factors are important directions to intervene in the process of RA.

In recent years, T-cell activation Rho GTPase-activating protein (TAGAP), a GTPase-activating protein specific for RhoA, has been found to be associated with the pathogenesis of several autoimmune diseases [7]. The ubiquitously expressed cytoplasmic protein RhoA, a target factor for TAGAP, serves as a prerequisite for the induction of adaptive T-cell responses [8, 9]. At the same time, rheumatoid arthritis is

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characterized by an imbalance between regulatory T cells and T-helper type 17 (Th17) cells [10], in which overproduction TH17 cells secrete pro-inflammatory factors that promote the development of RA [11]. Consequently, the role of TAGAP in RA deserves to be explored in depth.

In addition, NLRP3 inflammasome is also an influential factor in rheumatoid arthritis [12]. There is increasing evidence that NLRP3 inflammasome is associated with the pathogenesis of auto-inflammatory and auto-immune diseases [13], among which the role of NLRP3 inflammasome in gouty arthritis has been well documented [14]. A recent study found that NLRP3 is also associated with the differentiation of TH17 cells [15]. Hence, whether TAGAP can contribute to NLRP3 is also one of the focuses of this study.

The aim of this study was to clarify whether interference with TAGAP expression could have any impact on the course of RA and to investigate the association of the TAGAP gene with the relevant loci in RA. In our study, collagen-induced-arthritis (CIA) rat model and isolated $CD4^+$ T cells simulated the *in vivo* environment of RA and the cellular environment *in vitro*, and TAGAP gene expression was affected by TAGAP overexpression lentivirus or lentiviral vectors carrying TAGAP interference fragments to investigate the effect of TAGAP on RA progression and its mechanism of action.

Materials and methods

Animal

Six-week-old-specific pathogen-free (SPF) male Wistar rats (170–200 g) purchased from SPF Biotechnology (Beijing, China) were maintained at 24 ± 2 °C, 55 ± 5% humidity, and a 12 h light/dark cycle with *ad libitum* access to food and water ($n = 6$). Animal experiments were approved by the Animal Ethics Committee of Shaoxing People's Hospital (approval number: 2020-53). Animal experiments were conducted in strict accordance with international ethical regulations for laboratory animals.

Lentivirus transfection

The shRNA lentivirus specifically targeting the TAGAP (LV-TAGAP-shRNA), negative control lentivirus (LV-shRNA-NC or LV-OE-NC), and TAGAP overexpression (OE) lentivirus were purchased from GeneChem (GeneChem, China) and transfected with Lipofectamine 2000 (Thermo Fisher, China) according to the manufacturer's instructions. Change the original medium after 6 h of transfection to prevent the toxicity of the transfection reagents from making the cells poorly active.

CIA rat model construction and evaluation

RA was induced in rats under anesthesia by tail vein injection of bovine type II collagen (Chondrex, USA) emulsified in complete Freund's adjuvant (CFA). All rats were injected with an arthritis-inducing agent subcutaneously at the root of the tail on day 0, except for normal control (NC), which received saline injection. Two weeks later, we boosted the immunization with emulsified bovine type II collagen except for the NC group. A total of 24 rats were randomly allocated into four groups, with each group consisting of six rats. The random division was carried out by blindly numbering the rats and utilizing Excel, while strictly adhering to the principles of the 3R rules, which prioritize the use of the minimum number of animals. Rats of NC group were not treated in any way beside saline injection. CIA rats injected with saline were the model group (CIA). CIA rats injected with blank lentiviral vector (3 days/time) were CIA + LV-shRNA-NC group, and CIA rats injected with lentiviral vector with TAGAP silencing fragment (3 days/time) were CIA + LV-TAGAP shRNA group. Rats were given ankles injections every 3 days from day 15 to day 30.

After the second immunization, two independent observers unaware of the animal treatment checked the severity of arthritis in the rats once every 3 days. The scoring system is as follows [16]: 0, no signs of erythema or swelling; 1, erythema and mild swelling confined to the tarsal or ankle joints; 2, erythema and mild swelling extending from the ankle to the tarsus; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; 4, erythema and severe swelling including the ankle, paws and digits, or ankylosis of the limb.

On day 30, isoflurane anesthetized rats were executed by posterior cervical dislocation, and joint tissues and serum from each group were collected for further study. Hind limb ankles were fixed in 4% paraformaldehyde, decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution for 4 weeks and then placed in a graded ethanol solution for stepwise dehydration from low to high, then permeabilized with xylene and paraffin embedded for subsequent histopathological evaluation.

Cell isolation and processing

Normal rat spleen was isolated and used to prepare cell suspensions. Normal rat spleen was isolated and used to prepare cell suspensions. $CD4^+$ T cells were positively selected by magnetic bead sorting with magnetic-activated cell sorting separation columns. Cells were stimulated with Phorbol 12-myristate 13-acetate (PMA), Brefeldin A and ionomycin for 6 h at 37 °C and harvested for lentiviral transfection. According to the manufacturer's program, $CD4^+$ T cells were transfected with lentiviruses vector TAGAP-shRNA and lentiviruses vector overexpressing TAGAP for 72 h.

Histopathological analysis

To assess the inflammatory status of the ankle joint, hematoxylin & eosin (H&E) staining was used. Five micrometers thin slices of the ankle samples were cut, sequentially stained with H&E, and then resin-sealed for microscopic observation.

Ankle sections were stained with saffron O solid green to assess cartilage damage. The ankle samples were cut into 5 μm slices, dewaxed and rehydrated into freshly prepared Weigert staining solution, washed in distilled water and differentiated in acidic ethanol. The slices were first stained with fast green and Safranin O. The residual fast green was removed by washing with acetic acid solution and then washed with water. After dehydration, the stain was permeabilized with xylene and sealed with optical resin.

For immunohistochemistry, individual sections were stained using an anti-CD3 antibody (ThermoFisher, China) to identify $CD3^+$ T cells. Images were acquired using an optical microscope.

ELISA assay

Quantification of IL-1β, IL-17, and TNF-α in rat serum and cell culture supernatants was accomplished by IL-1β, IL-17, and TNF-α (Solarbio, China) ELISA kits, and measured at 450 nm using a microplate reader (ThermoFisher, China).

RT-qPCR

Total RNA was isolated using TRIzol reagent (ThermoFisher, China), dissolved in RNA-free H_2O, and stored at –80 °C. The cDNA was synthesized by RNA cDNA First Strand Synthesis Kit (TransGEN Biotech, China) according to manufacturer's instructions. The mRNA levels were quantified by SybrGreen qPCR Master Mix (TAKARA) and ABI Stepone plus system. The primer sequences are shown in Table 1. The qPCR was designed under the following conditions: after pre-denaturation at 95 °C for 3 min, a total of 45 cycles of denaturation at 95 °C for 7 s, annealing at 57 °C and extension at 72 °C for 15 s. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were repeated three times.

Western blot

Proteins were extracted from ankle synovial tissue and cell samples for western blot analysis, as described in previous experiments. Synovial tissue was minced with ophthalmic scissors, and for cell samples, cells were trypsinized and collected prior to lysis. Proteins were extracted from synovial tissue fragments and cells using ice-cold RIPA buffer (Beyotime, China) containing 10 μg/ml phosphatase inhibitor mixture and 10 μg/ml protease inhibitor mixture. The mixture was lysed on ice for 30 min and ultracentrifuged at 13 000 rpm for 10 min. Protein concentrations were determined using a bis-chondroitin acid (BCA) kit (Beyotime, China).

Protein extracts were loaded on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis for electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes (Merck-Millipore, Germany). The membranes were sealed with 5% skim milk (diluted with 0.05% Tween-20) for 1 h at room temperature. The membranes were incubated overnight in primary antibody dilutions consisting of rabbit anti-NLRP3 (1:1000; CST, USA), rabbit anti-RhoA (1:1000; CST, USA), and rabbit anti-glyceraldehyde phosphate phosphate dehydrogenase (GAPDH) (1:10 000; CST, USA). After the membranes were washed, they were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin (IgG) (1:10 000; CST, USA) for 2 h at room temperature. Finally, the ECL detection system displayed the results.

Flow cytometry

Tissue was gently ground with a 200-well cell filter, washed twice with phosphate-buffered saline (PBS), and then resuspended in RPMI-1640 medium. Cells were stained with anti-CD3-APC and anti-CD4-FITC in the dark at 4 °C. Th17 and Treg cells were then labeled with phaeoerythrin (PE)-conjugated anti-IL-17 and PE-conjugated anti-foxp3, respectively. FCM was performed using FITC Annexin V apoptosis detection kit I and by Cytoflex flow cytometer (Beckman Coulter, USA) according to the manufacturer's instructions and analyzed using FlowJo software (BD Pharmingen, USA). Un-labeled, florescence minus one and single stained controls were used as controls for gating and compensation.

Statistical analysis

All of the above experiments were repeated three times each. One-way analysis of variance test was used for comparison of multiple group means. Data are expressed as mean ± standard deviation. Analyses were performed using SPSS Statistics (version 22.0). $P < 0.05$ was considered significant.

Result

TAGAP interference treatment attenuates joint damage in CIA rats

To investigate the effect of TAGAP on RA, we constructed the CIA rat models. The mRNA expression of TAGAP in PBMC of CIA rats was detected by qPCR, and the expression of TAGAP in CIA rats was found to be much higher than that in normal rats (Fig. 1A), which indicated that TAGAP was highly expressed in CIA rats.

After TAGAP interference, paw redness and joint swelling were significantly reduced in the CIA + LV-TAGAP shRNA group compared to the CIA + LV-shRNA-NC group (Fig. 1B). In addition, the arthritis index showed that TAGAP interference significantly reduced joint inflammation starting on day 15 after TAGAP interference $^{*}P < 0.05$, and the difference with the CIA + LV-shRNA-NC group continued to be maintained. $^{***}P < 0.001$ (Fig. 1C).

To further assess the effect of TAGAP interference on synovial and cartilage destruction, a histopathological evaluation was performed. Cartilage damage, pannus formation, and inflammatory cell infiltration were observed in the CIA group of rats through H&E staining, whereas the rats injected with CIA + LV-TAGAP shRNA showed reduced cartilage damage, synovial proliferation, and pannus formation in the ankle joint. Additionally, Safranin-O staining (with red

Table 1. RT-qPCR primer sequences

mRNA	Forward pprimer 5′ to 3′	Reverse primer 5′ to 3′
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
TAGAP	TTCACCACAAAGACGGACAA	GAGAGGGCCCAGGTTCCAGA
TNF-α	CCAAGGCGCCACATCTCCCT	GCTTTCTGTGCTCATGGTGT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-1β	GAAGAAGAGCCCATCCTCTG	CTTGCACGTTGAGTTTGGGT
IL-10	GCTGGACAACATACTGCTAACC	ATTTCCGATAAGGCTTGGCAA
MMP-3	ACATGGAGACTTTGTCCCTTTTG	TTGGCTGAGTGGTAGAGTCCC
MMP-13	CTTCTTCTTGTTGAGCTGGACTC	CTGTGGAGGTCACTGTAGACT
BCL-2	CCGGGAGAACAGGGTATGAT	GCACAGCGGGCATTGGGTTG
IFN-γ	CCAACGCAAAGCAATACATGA	CTTGCACGTTGAGTTTGGGT

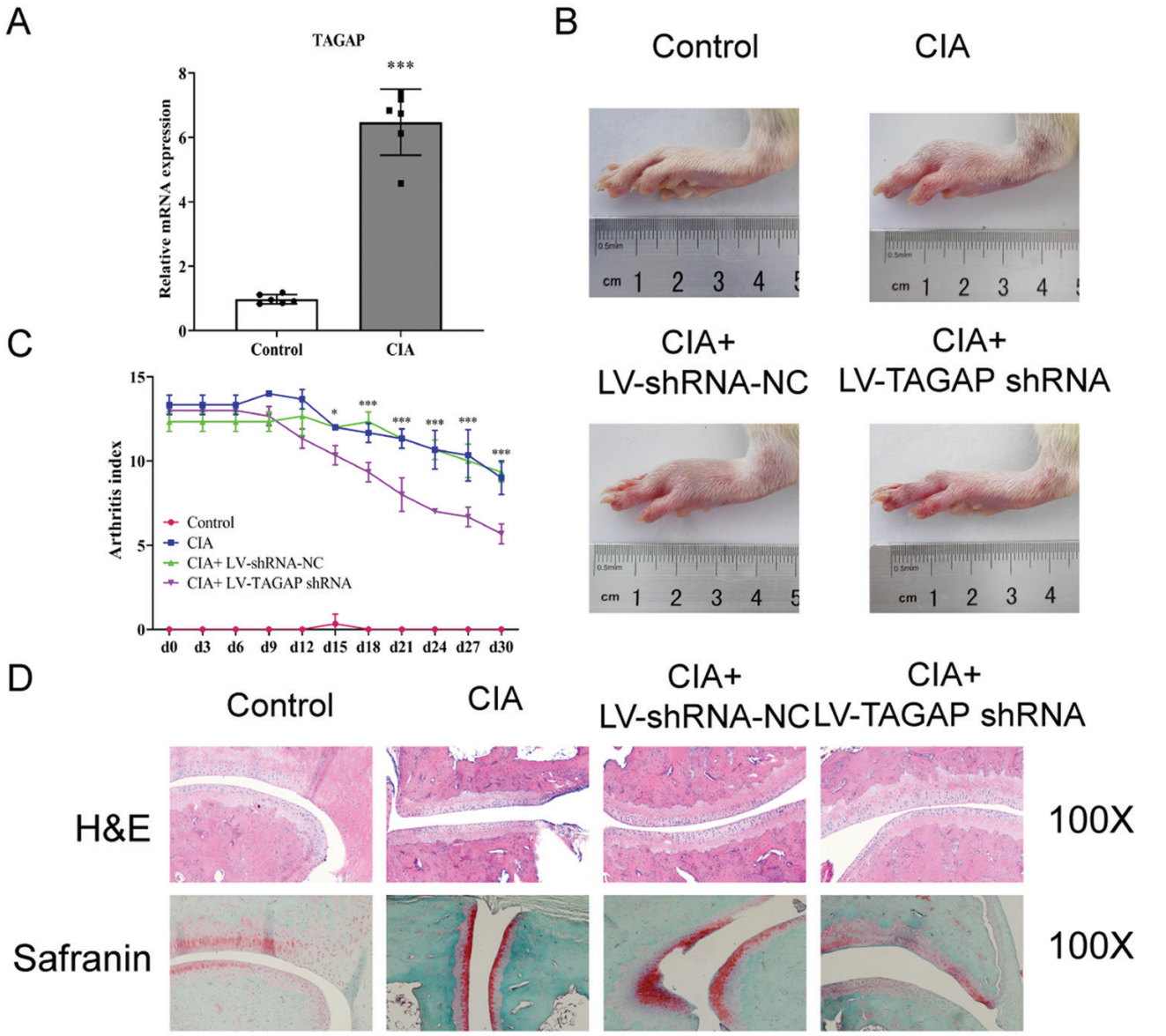

Figure 1. TAGAP interference attenuates ankle osteoarthritis in CIA rat models. (A) TAGAP expression in serum of CIA rat model ($n = 6$). (B) The degree of ankle and paw swelling in each group of rats on day 30. Our scoring system was as follows: 0, no signs of erythema or swelling; 1, erythema and mild swelling confined to the tarsal or ankle joints; 2, erythema and mild swelling extending from the ankle to the tarsus; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; 4, erythema and severe swelling including the ankle, paws and digits, or ankylosis of the limb ($n = 6$). (C) Trends in arthritis indices of rats in each group during administration. CIA-LV-TAGAP-shRNA vs. CIA-LV-shRNA-NC, $^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$. (D) H&E staining of the ankle joint of CIA rats (blue area indicates nucleus and cell membrane, red area indicates cytoplasm, and extracellular matrix) (×100). Safranin O/fast green staining of the ankle joint of CIA rats (red area indicates cartilage area; green area indicates bone tissue, muscle and collagen fibers) (×100). All experiments were repeated three times ($n = 3$). CIA: collagen-induced-arthritis, TAGAP: T-cell activation Rho GTPase activating protein

color representing cartilage) revealed that TAGAP interference can also alleviate the thinning and erosion of cartilage caused by RA (Fig. 1D). These results suggest that TAGAP interference was able to alleviate the progression of RA in CIA rats.

TAGAP interference reduces joint inflammation in CIA rats

Animal experiments revealed by ELISA that the levels of TNF-α, IL 1β, and IL 17 were significantly increased in the ankle synovial tissue as well as in the serum of the CIA rats model after collagen induction, while the expression of TNFα, IL-1β, and IL-17 were significantly downregulated after LV-TAGAP shRNA injection compared with the model group (Fig. 2A).

The mRNA of pro-inflammatory cytokines, matrix degrading enzymes, and anti-inflammatory cytokines were detected by RT-qPCR in the serum of rats in four groups. Compared with the model group, the pro-inflammatory factors (TNFα, IL-1β, IL-6), matrix degrading enzymes (MMP-3, MMP-13) were significantly decreased in the CIA + LV-TAGAP shRNA group, while the anti-inflammatory factor IL-10 was significantly increased. The above results indicated that TAGAP interference was beneficial to the recovery of joint inflammation in rats (Fig. 2B).

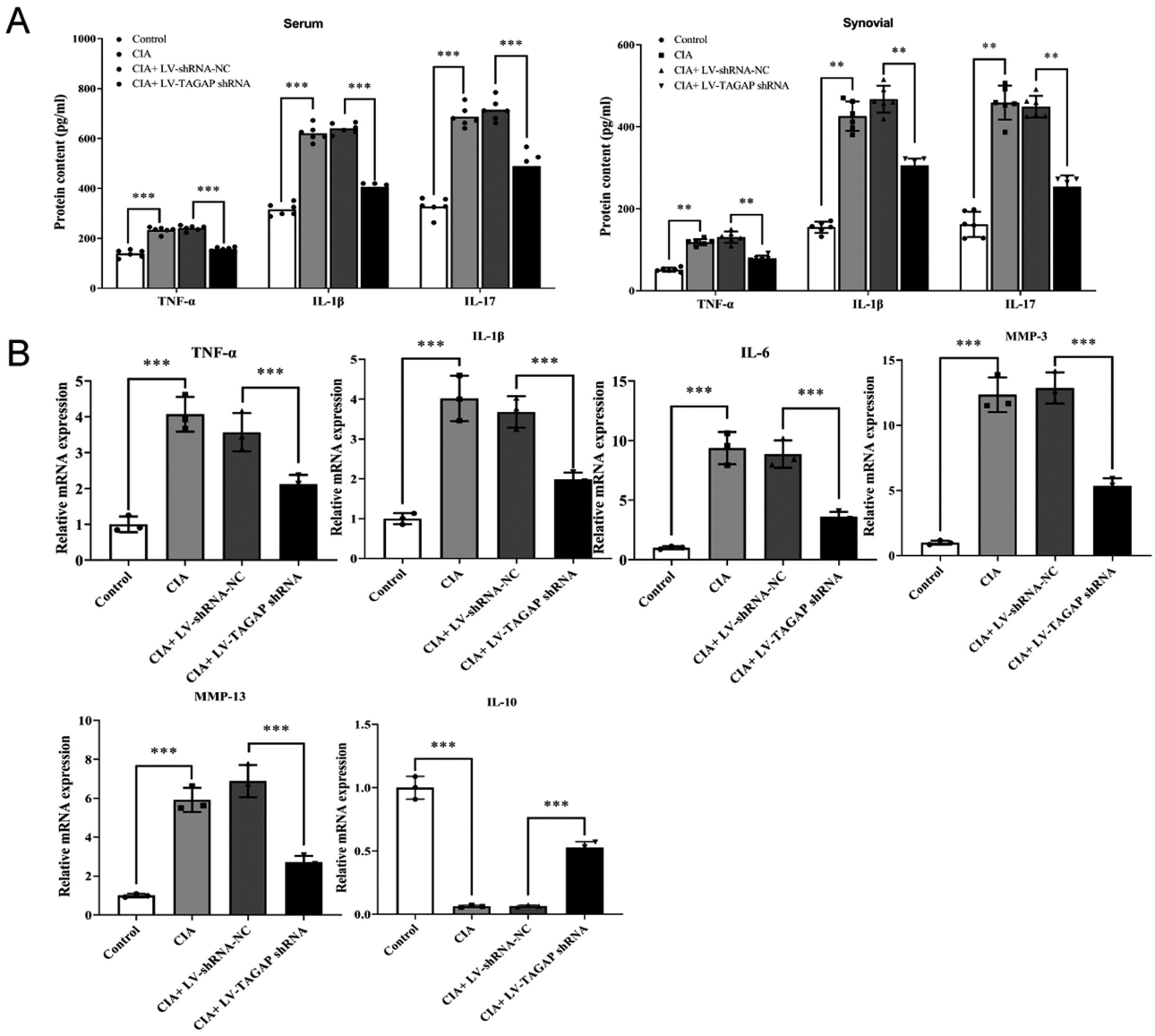

Figure 2. Inflammatory factor expression is affected in CIA rats under TAGAP interference. (A) The levels of TNFα, IL-1β, and IL-17 in serum and synovial tissues of rats. ***$P < 0.001$, $n = 6$. (B) The levels of TNFα, IL-6, IL-1β, MMP-3, MMP-13, and IL-10 in rat serum. ***$P < 0.001$, $n = 3$. All experiments were repeated three times. CIA: collagen-induced-arthritis, TAGAP: T-cell activation Rho GTPase activating protein

TAGAP interference treatment attenuates the inflammatory response of CD4⁺T cells

ELISA of the supernatant of stimulated CD4⁺ T cells revealed significant down-regulation of TNF-α, IL-1β, and IL-17 expression in the LV-TAGAP shRNA group and up-regulation in the LV-OE-TAGAP group (Fig. 3A). RT-qPCR detected the mRNA expression of BCL-2, IL-6, MMP-3, MMP-13 and IFN-γ in each group of cells, and found that the mRNA expression of the above factors was significantly down-regulated in the LV-TAGAP shRNA group and up-regulated in the LV-OE-TAGAP group compared with the control group (Fig. 3B). The above results suggested that TAGAP interference is capable of suppressing the inflammatory response of CD4⁺ T cells.

TAGAP interference inhibits the protein expression of RhoA and NLRP3

To explore the mechanism of TAGAP affecting RA progression, the protein content of RhoA and NLRP3 in synovial tissues was measured by WB. The results showed that the expression of RhoA and NLRP3 was significantly higher in the CIA group versus the CIA + LV-shRNA-NC group, and the expression was significantly lower in the CIA + LV-TAGAP-shRNA group compared with the CIA + LV-shRNA-NC group (Fig. 4A). Furthermore, in cellular experiments, the protein content of RhoA and NLRP3 in CD4⁺ T cells was found to decrease after TAGAP interference and to increase upon TAGAP overexpression (Fig. 4B).

TAGAP interference restores the balance of Th17 cells and Treg cells

Flow cytometry detection of TH17 and Treg cells in tissues revealed an increase in the proportion of Th17 cells ($CD3^+CD4^+IL\text{-}17^+$; Fig. 5A and B) and a decrease in the proportion of Treg cells ($CD4^+CD25^+FOXP3^+$) in the CIA group (Fig. 5C and D), whereas TAGAP interference resulted in a decrease in the number of Th17 cells and an increase in

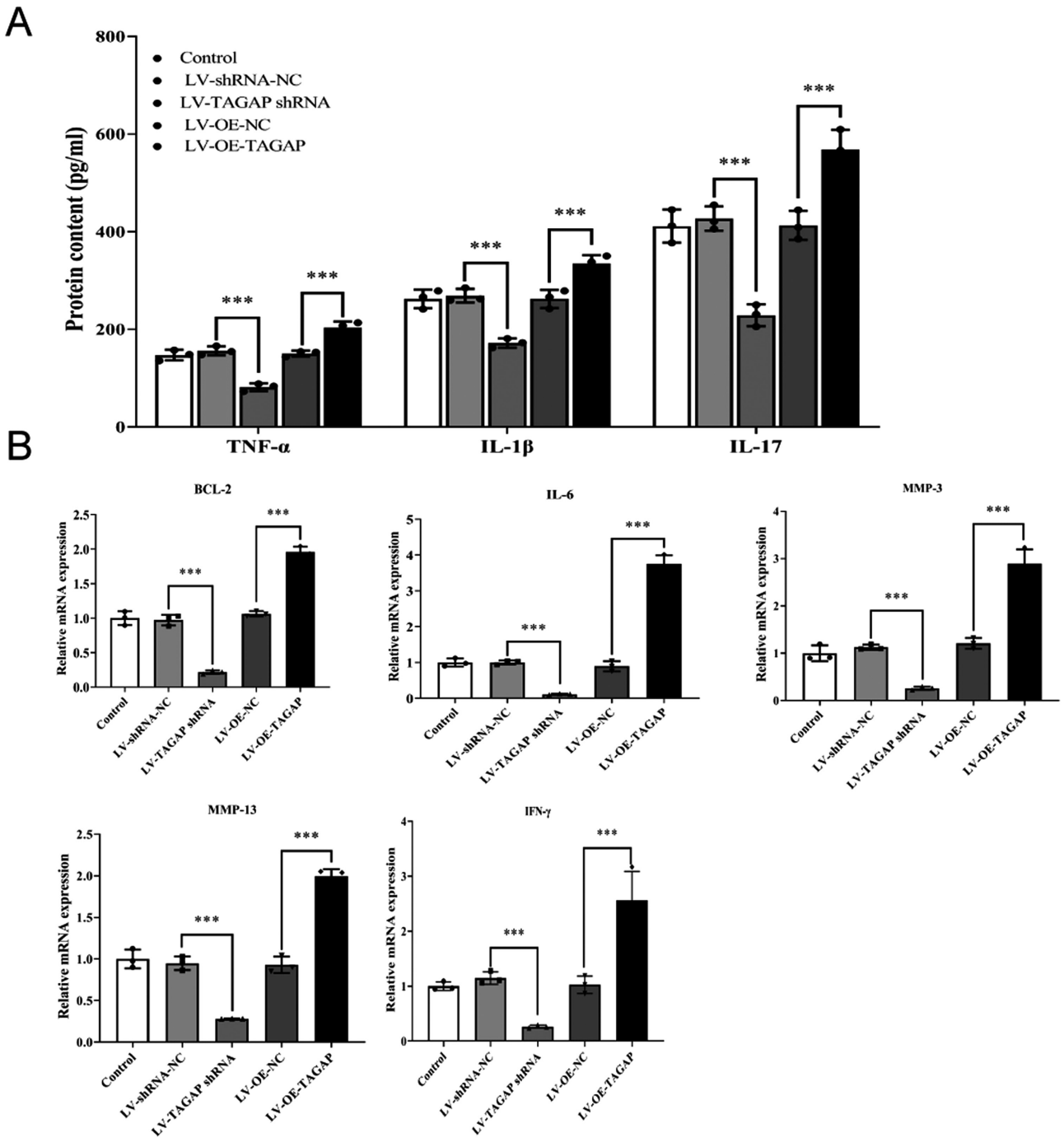

Figure 3. Decreased expression of inflammatory factors in CD4+ T cells after TAGAP interference. (A) The levels of TNFα, IL-1β, and IL-17 in the culture medium supernatant. $^{***}P < 0.001$, $n = 3$. (B) The levels of BCL-2, IL-6, MMP-3, MMP-13, and IFN-γ in the cells. $^{***}P < 0.001$, $n = 3$. All experiments were repeated three times. TAGAP: T-cell activation Rho GTPase activating protein

the number of Treg cells. The distribution of CD3+ T cells in the synovial was examined using immunohistochemistry. Compared to the normal group, the CIA group of rats exhibited a significant increase in the distribution of CD3+ T cells in the synovial, with some areas showing clusters of multiple CD3+ T cells. In contrast, in the synovial tissue of rats with TAGAP interference, the CD3+ T cells were significantly reduced (Fig. 5E). In *in vitro* experiments, Th17 differentiation levels were examined and found to be reduced after TAGAP interference and increased in the TAGAP overexpression group (Fig. 5F and G). The above results indicated that TAGAP interference inhibited the differentiation of TH17.

Discussion

RA, an autoimmune disease, presents as a chronic autoimmune arthritis of unknown cause, which makes it difficult to investigate the treatment of RA [17]. TAGAP is a protein that plays an inducing role in the differentiation of human CD4+ T cells into T cells. Previous studies have shown that TAGAP

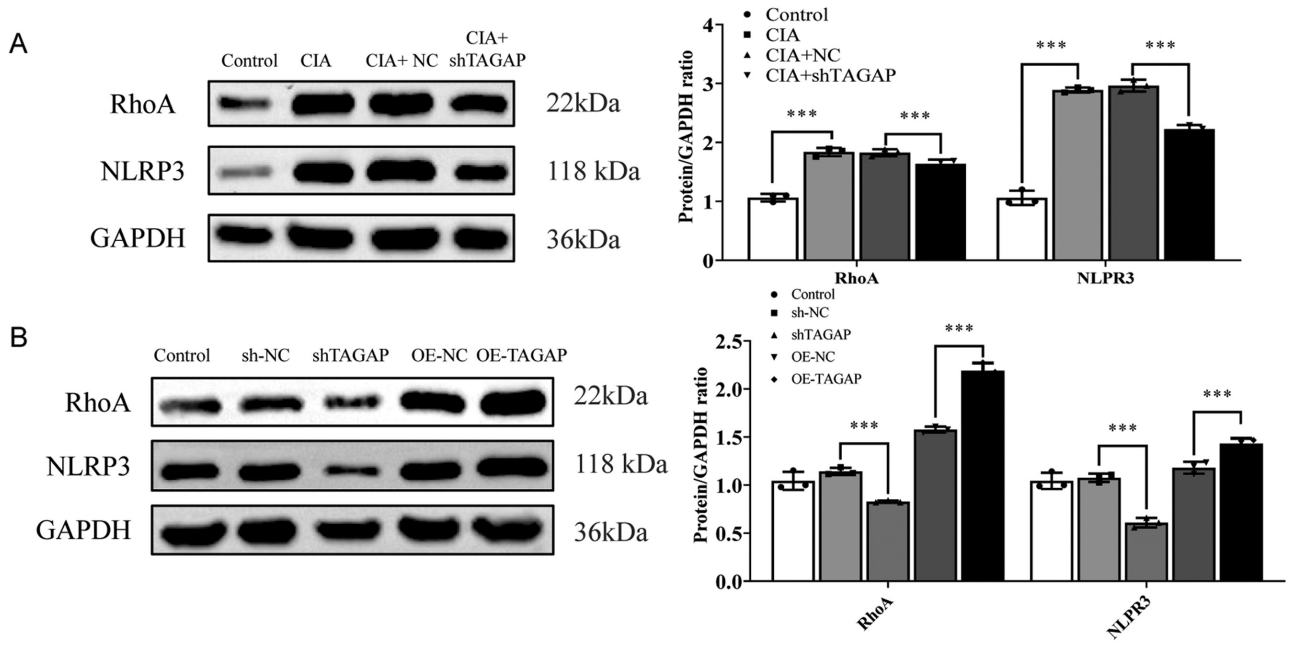

Figure 4. Protein content of RhoA and NLRP3 affected by TAGAP in CIA rats and CD4⁺ T cells. (A) Protein content of RhoA and NLRP3 in CIA rat models. $^{***}P < 0.001$, $n = 3$. (B) TAGAP interference or overexpression affects the protein content of RhoA and NLRP3 in CD4⁺ T cells. $^{***}P < 0.001$, $n = 3$. All experiments were repeated three times. CIA: collagen-induced-arthritis, TAGAP: T-cell activation Rho GTPase activating protein

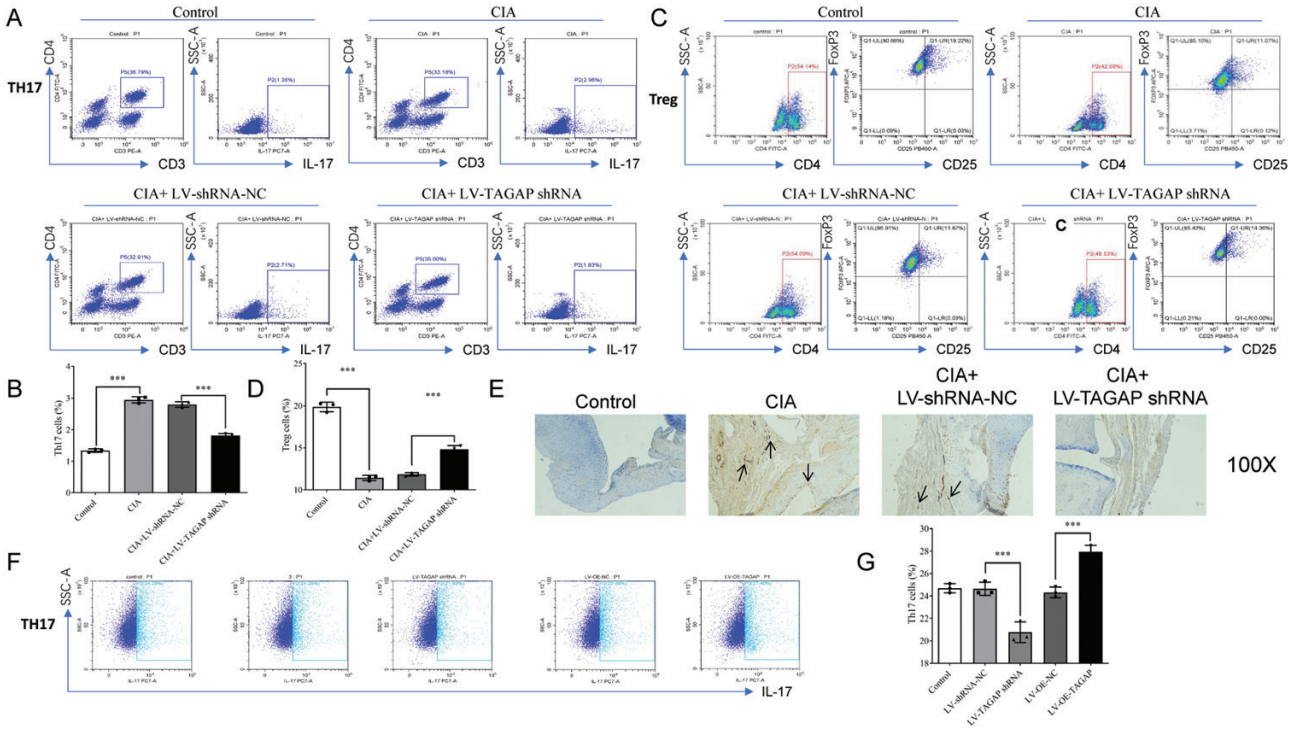

Figure 5. Restoration of Th17 cell and Treg cell differentiation after TAGAP interference. (A) Flow cytometry detection of Th17 cell differentiation in synovial tissue. (B) Quantification of TH17 cells in synovial tissue. $^{***}P < 0.001$. (C) Flow cytometry detection of Treg cell differentiation in synovial tissue. (D) Quantification of Treg cells in synovial tissue. $^{***}P < 0.001$. (E) The presence of CD3⁺ T cells in the synovial tissue was visualized through immunohistochemistry staining of CD3 (×100, CD3⁺ T cells aggregated into clusters are represented by black arrows.). (F) Flow cytometry detection of Th17 cell differentiation in CD4⁺ T cells. (G) Quantification of Treg cells in synovial tissue. $^{***}P < 0.001$, $n = 3$. All experiments were repeated three times. TAGAP: T-cell activation Rho GTPase activating protein

is upregulated in RA patients and arthritic mice [18]. In our investigation, utilizing the well-established CIA rat model [19], we observed heightened expression of TAGAP in CIA rats compared to the control group. Additionally, histological analysis revealed that interference with TAGAP can effectively mitigate RA symptoms. The inhibition of pro-inflammatory responses emerges as a pivotal therapeutic approach for RA [20, 21], supported by the observed decrease in the expression

of inflammatory mediators in the serum and synovium as TAGAP expression declines, while TAGAP overexpression leads to an increase in the expression of these inflammatory factors. *In vitro* experiments further substantiated the suppressive effect of TAGAP interference on the inflammatory response of CD4 + T cells. Finally, mechanistic investigations elucidated that TAGAP interference exerts its beneficial effects by inhibiting the expression of RhoA and NLRP3, thereby attenuating the differentiation of Th17 cells and ultimately contributing to the alleviation of RA symptoms. These findings shed light on the impact of TAGAP expression interference in RA and provide insights into its underlying mechanisms.

By constructing a CIA rat model, foot appearance and histopathological results showed that TAGAP interference reduced foot swelling, bone destruction, synovial inflammation, and cartilage erosion in CIA rats, indicating that TAGAP interference has a positive effect on slowing down RA development. Previous studies have found that TAGAP inhibits TCR signaling by disrupting the interaction between ZAP70 and RhoH, while weak TCR signaling promotes differentiation of TH17 cells [22]. Treg cells derived from the same precursor cells as TH17 cells can suppress the inflammatory response induced by TH17 cells, hence the regulation of TH17/Treg cell balance cannot be disregarded in the progression of RA. The results of this study showed that the percentage of Th17 cells was higher in the CIA rats than in the control group, while Treg cells showed the opposite trend. This is consistent with the results of previous studies in which clinical samples were tested [23]. To better visualize the effect of TAGAP interference on Th17 differentiation, we examined the differentiation of Th17/Treg in synovial tissues at the joints and examined the differentiation of $CD4^+$ T cells into TH17 *in vitro*. The results showed a decrease of TH17 cells and an increase of Treg cells in the CIA + LV-TAGAP shRNA group, demonstrating that the restoration of the TH17/Treg cell balance could be caused by TAGAP interference, which is consistent with the findings of previous studies [22]. In combination with foot appearance and histopathological assessment, it can be concluded that TAGAP interference was able to mitigate the development of RA by restoring TH17/Treg balance.

Th17 cells stimulate the initial inflammatory response by producing cytokines that are critical in the pathogenesis of RA [24–26], Some of these factors have the potential to become RA biomarkers, including tumor necrosis factor (TNF) [27] and IL 17 [28]. ELISA experiments revealed that the relative expression levels of inflammatory factors TNFα, IL-1β, and IL-17, as well as the relative mRNA expression levels of pro-inflammatory cytokines (TNFα, IL-6, and IL-1β) and matrix degrading enzymes (MMP-3 and MMP-13) in the joint by QPCR were significantly reduced after TAGAP interference, while the expression of anti-inflammatory cytokine IL-10 was significantly increased. The interference of TAGAP was shown to inhibit the inflammatory response in RA to mitigate the development of RA.

Previous studies have reported that NLRP3 has a regulatory role in the balance of Th17/Treg cells [15], suggesting the importance of the NLRP3 inflammasome in the innate and adaptive immune response, moreover, studies have reported that activation of the NLRP3 inflammasome pathway is associated with RA [29, 30]. NLRP3 inflammasome have been reported to be activated in the synovium of RA mice, and treatment with selective NLRP3 inhibitors reduced the symptoms of arthritis and cartilage destruction [31–33]. Therefore, NLRP3 was examined in rat synovial tissue along with *in vitro* $CD4^+$ T cells, where it was found that NLRP3 levels were significantly elevated in CIA rats compared to control group, while notably TAGAP interference caused a significant decrease in NLRP3 protein levels. In addition, RhoA, the direct target of TAGAP, was significantly reduced after TAGAP interference as originally expected. The above results suggest that TAGAP may alleviate RA by inhibiting NLRP3 and RhoA. This finding enriches the factors influencing the role of NLPR3 in RA. However, this study has a limitation in that it does not directly demonstrate the involvement of NLPR3 and RhoA in mediating TH17 cell differentiation in the context of RA. Nonetheless, previous studies have already established the role of NLPR3 and RhoA in regulating Th17 cell differentiation [32, 34], which provides some support for our conclusions.

To the best of our knowledge, this study represents the pioneering investigation into the role and underlying mechanism of TAGAP in the context of RA. In summary, our research commenced with an exploration of T-cell differentiation in RA and yielded compelling evidence of heightened TAGAP expression in CIA rats. Moreover, our findings unequivocally demonstrate that interference with TAGAP hampers the progression of RA and curtails the associated inflammatory response. Mechanistically, this interference exerts inhibitory effects on NLRP3 and RhoA expression, leading to a reduction in Th17 cell differentiation and ultimately ameliorating the severity of RA. These seminal findings establish a novel experimental foundation for targeting TAGAP as a therapeutic approach for RA and lay the theoretical groundwork for the future development and application of TAGAP antagonists as promising RA treatments.

Acknowledgements

Not applicable.

Ethical Approval

Animal experiments were approved by the Animal Ethics Committee of Shaoxing People's Hospital (approval number:2020-53).

Conflict of 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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Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

H.-g.S.: Resources, Methodology and wrote the first draft of the manuscript. Q.J.: Made animal models and performed behavioral studies. W.-j.F., X.-y.S.: Performed data analysis. Z.-w.W.: Data curation. X.W.: Funded acquisition, Project Administration and Revised the final draft.

Permission to Reproduce

Not applicable.

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