MicroRNA-143-3p alleviates murine collagen-induced arthritis by polarizing naive CD4+T cells into Treg cells

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Abstract

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease with the decreasing proportion of regulatory T (Treg) cells. Previous studies have shown that microRNAs (miRNAs, miR) act as key regulators of Treg cells. In this study, we assessed the involvement of miR-143-3p on Treg cells differentiation and function in the RA progress. We reported that the expression of miR-143-3p has been negatively associated with RA disease activity, and actively correlated with anti-inflammatory cytokine IL-10, which was secreted by Treg cells. In vitro, miR-143-3p expression in the CD4+T cells contributed to the upregulation of forkhead box protein 3 (Foxp3), which was the characteristic transcription factor of Treg cells. Notably, miR-143-3p mimics treatment markedly upregulated the frequency of Treg cells in vivo, effectively prevented CIA development and significantly inhibited inflammation in mice. Altogether, we proposed that MiR-143-3p can alleviate CIA by polarizing naive CD4+T cells into Treg cells, which warrants miR-143-3p as a target for the new therapeutic strategy of Treg-deficiency autoimmune diseases such as RA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by chronic inflammatory cell infiltration and pannus formation in synovial tissues that lead to the destruction of articular cartilage and bone[1,2]. The inflamed synovium is hallmarked by inflammatory cells infiltration involving innate immune cells and adaptive cells (B cells and T cells)[3]. Although the pathogenesis of RA remains obscure, it has been accepted that the abnormal activation of $CD4^+$T helper (Th) cells can cause the initiation and progression of RA[4]. Plasticity of $CD4^+$T cells, the ability to differentiate into specific subsets, is essential for maintaining immune homeostasis[5]. In the stimulation of immunoregulatory cytokines such as TGF-β and IL-2, naïve $CD4^+$ T cells could differentiate into Treg cells, which have a vital role in immune tolerance by suppressing pathogenic T cell responses[6]. Recently, quantitative and functional defects of Treg cells have been determined in the peripheral blood (PB) and synovial fluid of RA patients[7,8]. Treg cells are well-characterized for their specific expression of and Foxp3, and can secrete anti-inflammatory cytokines such as TGF-β, IL-10, IL-35, which inhibit effector T cells differentiation and function[9,10]. The modulation on Treg cells differentiation could be helpful for the treatment of RA.

The role of Treg cells in RA is clear, but the exact mechanisms were still being worked out. MicroRNAs (miRNAs), considered as a class of meticulous regulator for targeting messenger RNA (mRNA). They impact the expression of functional crosstalk genes and participate in the etiologic of inflammatory diseases[11,12]. Mounting evidence suggests that miRNAs are irreplaceable regulators on part of Treg cell differentiation and/or function. For instance, miR-146a and miR-155 was downregulated in Treg cells of RA patients, and correlated with joint inflammation[13]. MiR-342 was confirmed to control Treg regulatory functions via

targeting the mTORC2 component, Rictor[14]. Simultaneously, miR-142-3p was reported to target Tet2 and result in impaired Treg stability in islet autoimmunity[15]. MiR-34a could attenuate Foxp3 gene expression via targeting their 3' untranslated regions (3' UTR) in collagen induced arthritis (CIA) mice[16]. However, whether other miRNAs participate in regulating Treg cell differentiation and/or function and how these miRNAs are regulated need further investigation.

Our previous studies have also found the abnormal expression of miR-143-3p in PB of RA patients by miRCURY^{TM} LNA Array[17]. And in the present study, we demonstrated that miR-143-3p attained the highest negative correlation with RA disease activity, and it positively correlated with IL-10 in RA. We then designed the in vivo and in vitro experiments and verified the effect of miR-143-3p on Treg cell differentiation. Our data suggested that up-regulation of miR-143-3p markedly ameliorates CIA pathogenesis by inducing Treg cell differentiation. Overall, this study explored the role of miR-143-3p on Treg cell differentiation in RA pathogenies, which identified a promising new target for curing RA.

Materials and methods

RA patients and control subjects

Sera and clinical details from 33 patients with RA who fulfilled the 1987 American College of Rheumatology (ACR)/2010 European League Against Rheumatism (EULAR) classification criteria for RA were obtained without any treatment. All patients had active disease with Disease Activity Score at 28 joints (DAS28) > 3.2. They were recruited from the Department of Rheumatology, Jiangsu Province Hospital of Chinese Medicine. The study protocol was approved by the institutional review board (IRB) of the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (process number: 2016NL-KS14) and the study complied the principles of the Declaration of Helsinki. All participants read the study information sheet and signed the partake consent.

Clinical data about the disease, physician, and patient global assessment and DAS28 score were assessed. Clinical characteristics of the patients and the 24 healthy controls (HCs) were shown in**Table 1** . Blood was collected for ELISA and miRNA analysis.

Table 1. Characteristics of patients with RA and HCs

	Moderate RA (n=15)	Severe RA (n=18)	Healthy Controls (n=24)
Sex (Female %)	76.92	88.89	79.17
Age (years)	49.23±3.06	58.56±2.71	59.32±1.64
Tender joint count	2.46±0.37	8.00±1.00	NA
Swollen joint count	2.08±0.33	7.50±0.97	NA
ESR (mm/h)	37.62±5.27	73.94±7.14	NA
CRP (mg/L)	19.69±5.19	57.78±14.34	NA
DAS28	4.30±0.17	6.02±0.14	NA
RF positivity (%)	76.92	94.44	NA
IgG (g/L)	13.62±0.39	18.01±2.14	NA
IgA (g/L)	2.65±0.22	3.53±0.30	NA
IgM (g/L)	1.43±0.17	1.48±0.14	NA

Data are expressed as the mean ± SEM. ESR, erythrocyte sedimentation rate; CRP, C reactive protein; DAS28, Disease Activity Score of 28 joints; RF, rheumatoid factor; IgG, Immuno-globulin-G; IgA, Immunoglobulin-A; IgM, Immunoglobulin-M.

Separation of peripheral blood CD4$^+$ T cells

For human samples, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (TBD sciences, LTS1077). The separated PBMCs were washed twice with BD IMagTM buffer

and then incubated with BD IMagTM Anti-Human CD4 Particles (BD Biosciences, San Jose, CA) on ice for 30 min. 1 mL BD IMagTM buffer containing 10^7 cells were placed in the magnet for 8 min, and then $CD4^+$T cells were collected.

Animals

Male DBA/1J mice (6-8 weeks old) were purchased from Gempharmatech Co., Ltd [Changzhou, China; Animal license number: SCXK (Su) 2016-0010], and male C57BL/6 mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd [Beijing, China; Animal license number: SCXK (Jing) 2016-0006]. Mice were kept in specific-pathogen-free conditions. All mice experiments were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine (No. 201901A022; No. 202005A022).

Separation, transfection and differentiation of naive $CD4^+$ T cells

Murine $CD4^+$ T cells were obtained from the splenocytes of C57BL/6 mice with a mouse naive $CD4^+$ T cell isolation kit, according to the manufacturer's instructions (Miltenyi Biotec, Gladbach, Germany). Cells were stimulated in vitro with anti-CD3 and anti-CD28 antibodies (BD Pharmingen, San Diego, CA) (both for 2 μg/ml) in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin. ShRNA/lentivirus were purchased from Genepharma (Shanghai, P.R. China) to achieve the overexpression or knockdown of miR-143-3p. Under the stimulation by anti-CD3 and anti-CD28 antibody, the naïve $CD4^+$T cells were infected by lentiviral-conditioned media following the manufacturer's instructions. To induce the differentiation of Treg cells, naive $CD4^+$ T cells were supplemented with 5 ng/mL TGF-β (R&D Systems, Minneapolis, USA) and 80 ng/mL IL-2 (R&D Systems, Minneapolis, USA) after 12 h transfection[18]. Cells were analyzed by RT–PCR and flow cytometry.

Induction of CIA and miR-143-3p treatment

The CIA mice model was prepared as previously described[19,20]. DBA/1J mice were used for CIA induction by bovine type II collagen (CII) Chondrex, Inc., Washington, DC, USA), acetic acid, and Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA). The day of the first immunization was determined on day 0. On day 21, 0.1 mL CII emulsified with incomplete Freund's adjuvant (IFA) (final concentration of 1 mg/mL) was i.d. in the tail, proximally to the primary injection site, to enhance immunization.

A total of 24 DBA/1J mice were randomly divided into 4 groups as follows (n = 6 per group): control group, normal mice were injected with saline (1 mL/kg, i.v.); model group, CIA mice administered with saline (1 mL/kg, i.v.); control mimics group, CIA mice were injected intravenously with a 200 μL mixture of EntransterTM-in vivo (25 μL, Engreen Biosystem Co, Ltd., Beijing, P.R. China) and control mimics (50 μg); miR-143-3p mimics group, CIA mice were injected with a 200 μL mixture of EntransterTM-in vivo (25 μL) and miR-26b-5p mimics (50 μg). MiR-143-3p mimics or the control substances were administered at day 28 and day 35 after the first immunization[21]. The paw thickness and arthritis severity were detected at day 22, 26, 30, 34, 38, 42. Mice were sacrificed on day 44, and spleens, lymph nodes, plasma, and keen joints were obtained for further studies.

Arthritis scores

The paw thickness was blindly measured with the caliper, and arthritis severity was scored every 4 days after the second immunization. Arthritis severity was scored 0-4[22]. The maximum scores per mice were 16 (4 points × 4 paws).

Histologic analysis

Soaked the knee joints in 4 % paraformaldehyde for fixation, and then soaked in EDTA to decalcify them. Embedded it in paraffin for slices, and then stained with hematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP). Histology scores were evaluated as published articles[23,24] described, and determined by semiquantitative scoring systems.

Enzyme-linked immunosorbent assay (ELISA)

PB was collected from patients, HCs and mice in heparin sodium to centrifuge plasma. The levels of IFN-γ (Catalog #EK180HS), IL-4 (Catalog #EK104HS), IL-10 (Catalog #EK110HS) and IL-17A (Catalog #EK117HS) were determined by Human High Sensitivity ELISA kit (MultiSciences Biotech Co., Ltd., Hangzhou, China), and the leves of IL-1β (Catalog #EK201BHS), IL-6 (Catalog #EK206HS), TNF-α (Catalog #EK282HS), IL-10 (Catalog #EK210/3), IL-17A (Catalog #EK217HS) were detected by Mouse High Sensitivity ELISA kits or Mouse ELISA kits (MultiSciences Biotech Co., Ltd., Hangzhou, China). All ELISA procedures subjected to the manufacturer's instructions.

Isolation of total RNA and quantitative real-time PCR

Total RNA was extracted by TRIzol TM Reagent (Ambion, Texas, USA). Concentration and purity of RNA were determined using NanoDrop Microvolume spectrophotometer (Thermo ScientificTM, USA). For miRNA analysis, 500 ng of total RNA was performed with miRNA 1st Strand cDNA Synthesis Kits (by stem-loop) (Vazyme, Nanjing, P.R. China) to generate cDNA. For other mRNA analysis, cDNA was generated by reverse transcription kit HiScript Q RT SuperMix (Vazyme, Nanjing, P.R. China). Quantification of mRNA was done by by real-time PCR performed on resulting cDNA via miRNA Universal SYBR qPCR Master Mix or ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and an ABI 7500 thermocycler (Applied Biosystems Life Technologies, Foster City, CA, USA). The housekeeping gene U6 and GAPDH were used as standards for the normalization of mRNA expression values. The primers (Sangon Biotech Co., Ltd., Shanghai, P.R. China) used in this study were listed in**Table 2** :

Table 2. Primers used for quantitative real-time PCR

Name	Primers	Sequence 5'-3'
Has-U6	Forward	GCTTCGGCAGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGTCAT
Has-miR-143-3p	Forward	CGCGTGAGATGAAGCACTG
	Reverse	AGTGCAGGGTCCGAGGTATT
Mmu-U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
Mmu-miR-143-3p	Forward	GGGTGAGATGAAGCACTG
	Reverse	CAGTGCGTGTCGTGGAGT
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	TGTAGACCATGTAGTTGAGGTCA
Foxp3	Forward	CACCTATGCCACCCTTATCCG
	Reverse	CATGCGAGTAAACCAATGGTAGA

Intracellular staining and flow cytometry

As previously described[25,26], splenocytes and PBMCs from CIA mice were washed with PBS. For the analysis of Treg cells, cells were stained with anti-CD4-FITC (BD Bioscience), anti-CD25-PE-CY7 (BD Bioscience) and anti-Foxp3-PE (BD Bioscience). For analysis and gating, we set up auxiliary staining groups including no staining, single staining, isotype staining. Finally, data were acquired on flow cytometry (Beckman FC-500, USA).

Statistical analysis

Data were presented as mean ± SEM and statistically significant were considered if $p < 0.05$. One-way ANOVA with Dunnett's post-hoc test, Spearman's rank correlation, Linear regression were performed for statistical comparisons via IBM SPSS Statistics 19.0 and GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Differential expression of miR-143-3p is associated with disease activity in the peripheral blood CD4$^+$T cells of RA patients

To explore whether miR-143-3p participated in the pathogenesis of RA, the levels of miR-143-3p in the CD4$^+$T cells from PBMCs of RA patients and HCs were analyzed by qPCR. As miR-143-3p has been reported to show different expression in different individuals with RA[17,27,28], we speculate whether the difference of disease severity or not affected the expression of miR-143-3p. Hence, patients were classified according to Disease Activity Score-28 (DAS28). 18 patients had severe disease activity (DAS28¿5.1), 13 patients had moderate disease activity (DAS28 3.2-5.1). The expression of miR-143-3p in peripheral blood CD4$^+$T cells was significantly higher in patients with moderate RA than in HC, while levels of miR-143-3p were significantly lower in patients with severe RA in comparison with moderate RA (Figure 1D). Remarkably, based on Pearson's correlation analysis, the expression of miR-143-3p was negatively correlated with the DAS28 (r=-0.5530; p=0.0006) and ESR (r =-0.3425, P=0.0296) (Figure 1E-F), which were considered as markers of disease activity in RA. These data showed that lower expression of miR-143-3p in peripheral blood CD4$^+$T cells of RA patients have higher disease activity.

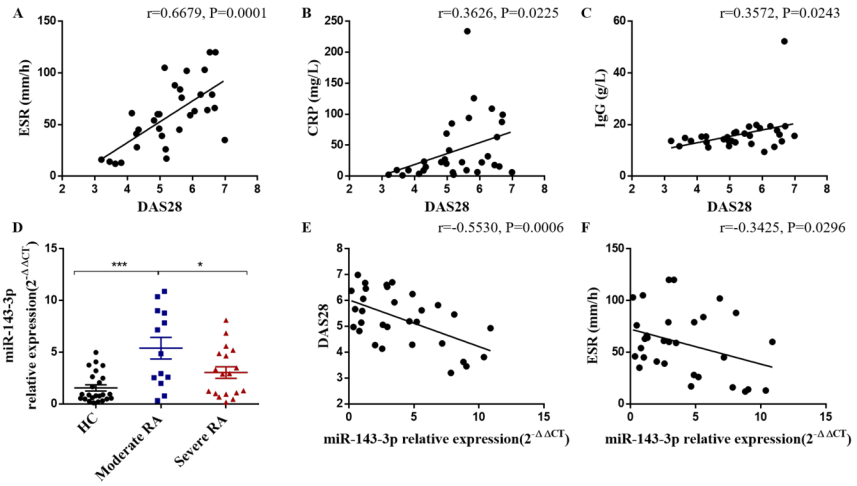

Figure 1. The expression of miR-143-3p in peripheral blood CD4$^+$T cells of RA patients and its correlation with DAS28 and ESR levels.

(A-C) The correlation between DAS28 and ESR (n=31, r=0.6681, P=0.0001) and CRP (n=31, r=0.3623, P=0.0226) and IgG levels (n=31, r=0.3568, P=0.0244). The Pearson correlation coefficient was determined.

(D) Quantification of miR-143-3p mRNA in peripheral blood CD4$^+$T cells of moderate RA patients (n=13) and moderate RA patients (n=18) and healthy controls (n=24). Data were expressed as the mean ± SEM. * $p < 0.05$, **$p < 0.01$, by one-way ANOVA with Dunnett's post-hoc test.

(E-F) The correlation between miR-143-3p expression and DAS28 (n=31, r=-0.5534, ***, P=0.0006) and ESR levels (n=31, r=-0.3422, *, P=0.0297). The Pearson correlation coefficient was determined.

MiR-143-3p expression is positively correlated with Treg-related cytokine IL-10

Negative correlation of miR-143-3p with markers of disease activity indicates the potential protective effect in RA. Due to aberrant activation of CD4$^+$ T helper (Th) cells considered as one of the crucial causations in the initiation and perpetuation process of RA, we collected some Th-related cytokine in plasma of patients by ELISA, such as IFN-γ, IL-4, IL-10, IL-17A, which predominantly secreted by Th1, Th2, Treg, and Th17 cells, to further evaluate the role of miR-143-3p in the progression of RA. Compared with healthy controls, the levels of secreted IFN-γ and IL-17A were increased in a degree-dependent manner (Figure 2A, D).

And more notably, the level of secreted IL-4 and IL-10 in plasma was significantly higher in patients with moderate RA than in HC, while expression of IL-4 and IL-10 was slightly reduced in patients with severe RA in comparison with moderate RA (Figure 2B-C), whose changing trend was similar to miR-143-3p in peripheral blood $CD4^+$T cells. We also observed the levels of IL-4 (r=-0.3863, P=0.0159) and IL-10 (r=-0.3256, P=0.0369) were inversely correlated with the DAS28, which is consistent with the characteristics of IL-4 and IL-10 as anti-inflammatory factors in inhibiting inflammation (Figure 2E-H). Importantly, the results of further analysis showed that the expression of miR-143-3p was positively correlated IL-4 (r=0.3239, P=0.0377) and IL-10 (r=0.3443, P=0.0290). These results indicated that there was a correlation between miR-143-3p and Th2 or Treg cells. Recent bioinformatics analysis has obtained miR-143-3p may contribute to the Foxp3 signaling pathway[29,30]. Thus, we concentrated on the effect of miR-143-3p on Treg cells and conduct the further study.

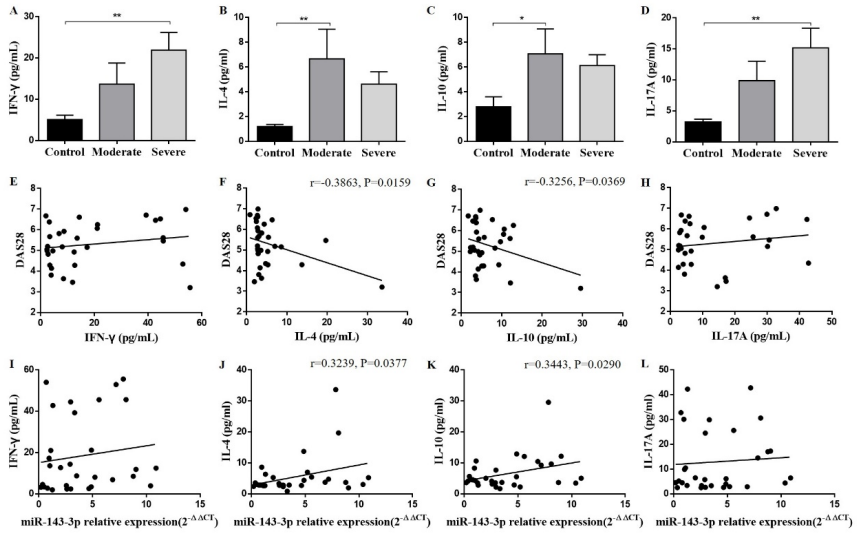

Figure 2. The levels of Th-related cytokines in plasma of RA patients and its correlation with DAS28 and miR-143-3p.

(A-D) The levels of IFN-γ, IL-4, IL-10 and IL-17A in plasma of moderate patients (n=13) and severe patients (n=18) were detected by ELISA. Data were expressed as the mean ± SEM. * $p < 0.05$, **$p < 0.01$, by one-way ANOVA with Dunnett's post-hoc test.

(E-H) The correlation between IFN-γ, IL-4 (n=31, r=-0.3863, P=0.0159), IL-10 (n=31, r=-0.3256, P=0.0369), IL-17A and DAS28 levels.

(I-L) The correlation between miR-143-3p expression and IFN-γ, IL-4 (n=31, r=0.3239, P=0.0377), IL-10 (n=31, r=0.3443, P=0.0290), IL-17A. The Pearson correlation coefficient was determined.

Down-regulation of miR-143-3p and decrease of Treg cells in CIA mice

To further confirm our findings, CIA model mice, which has a similar pathological mechanism to that of human RA[20,31], were successfully established to evaluate the relationship between miR-143-3p and Treg cells in vivo. Compared to those in WT mice, the arthritis score, paw swelling, histopathologic scores, TRAP-positive multinucleated cells, and inflammatory cytokine expression in CIA mice markedly increased (Figure 3-4). While a notable decline in the expression of miR-143-3p was detected in the splenic $CD4^+$T cells of CIA mice (Figure 3A). As reported in previous studies[32], the differentiation of Treg cells both in blood and spleen and the expression of Foxp3 in spleen and axillary lymph nodes (ALNs) markedly decreased in CIA mice compared to the control (Figure 5). These results suggest a possible positive effect of miR-143-3p on Treg differentiation and RA pathogenesis.

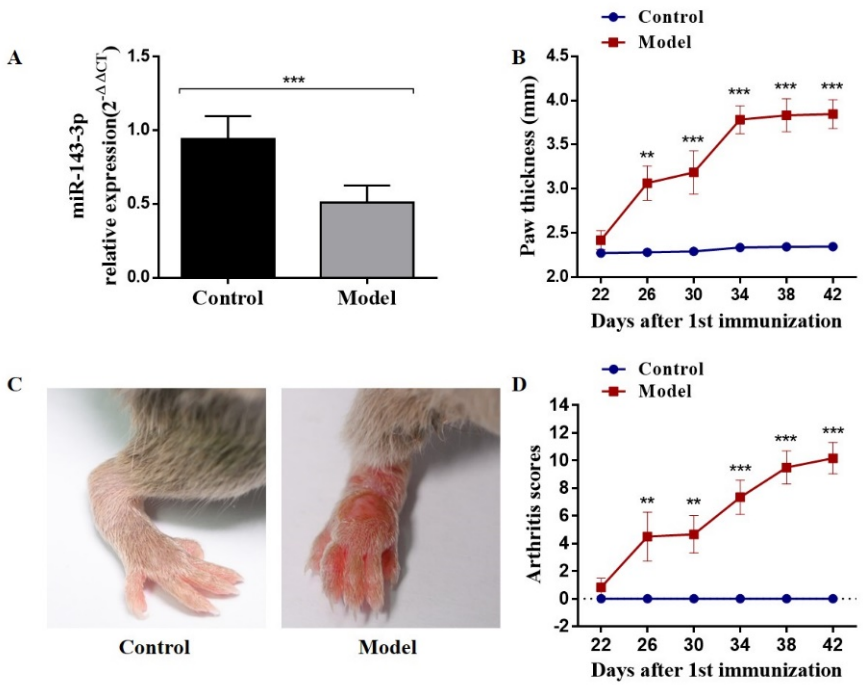

Figure 3. The expression of miR-143-3p and clinical severity of CIA mice.

DBA/1J mice were immunized with bovine type II collagen emulsified in complete Freund adjuvant and incomplete Freund adjuvant.

(A) MiR-143-3p expression in the splenic $CD4^{+}$T cells of control and CIA mice detected by qPCR analysis. Data were expressed as the mean ± SEM (n=6). *$p < 0.05$, and ***$p < 0.001$ compared with the control group by Student's t test.

(B-D) Mean arthritis score and paw thickness of control and CIA mice. Data were expressed as the mean +- SEM (n=6). * $p < 0.05$, **$p < 0.01$, by one-way ANOVA with Dunnett's post-hoc test.

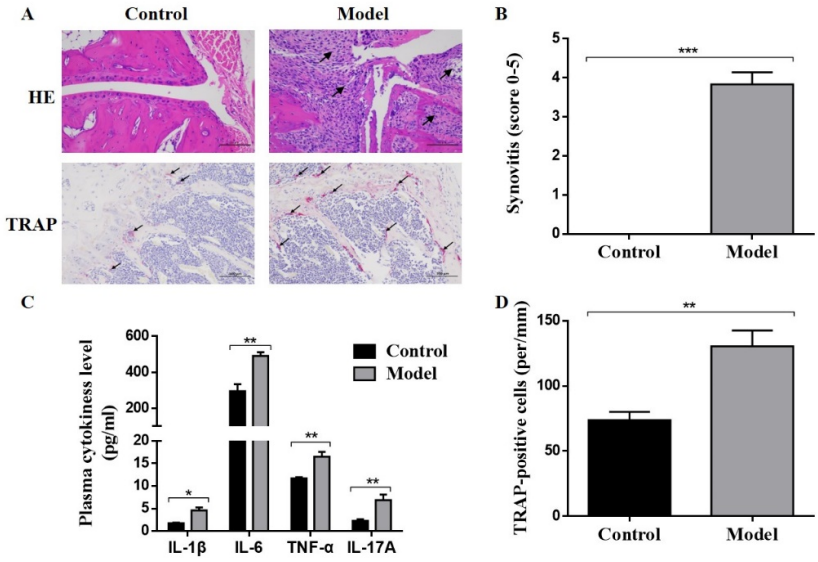

Figure 4. The histopathological changes and the inflammatory-related cytokine expression in CIA mice.

(A) Representative histopathological characteristics of CIA mouse joint. HE staining of joints of CIA mice showed inflammatory cell infiltration (black arrow). TRAP staining showed the activation of osteoclasts (black arrow) of mouse joints.

(B) Histopathologic indices of synovitis in the knee joints were determined using semiquantitative scoring systems (0-5 for the extent of infiltration by inflammatory cells into the synovium).

(C) The plasma levels of IL-1β, IL-6, TNF-α, IL-17A in CIA mice measured by ELISA on the 44th day after the first immunization.

(D) The number of TRAP-positive cells of CIA mouse joints.

Data were expressed as the mean ± SEM (n=6). * $p < 0.05$, **$p < 0.01$ compared with the control group by Student's t test.

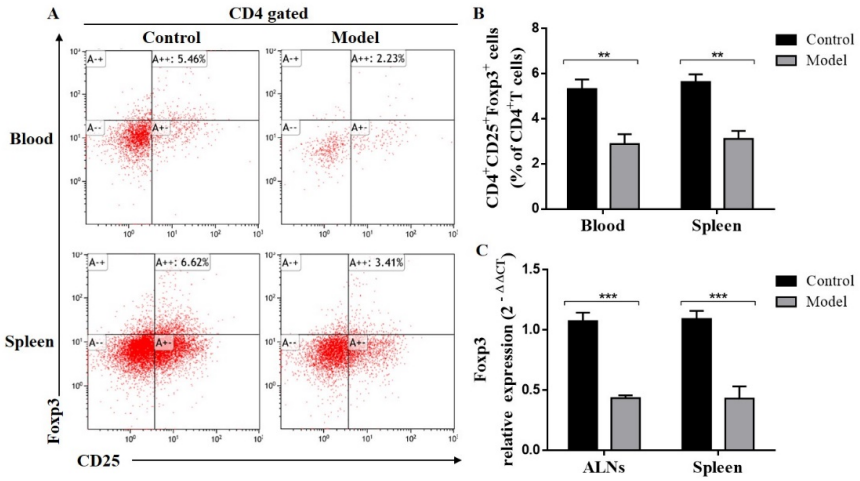

Figure 5. The percentage of Treg cells and the expression of Foxp3 in CD4$^+$T cells from CIA mice.

(A) Representative dot plots of CD4$^+$CD25$^+$Foxp3$^+$cells in blood and spleen of control and CIA mice.

(B) The percentage of Treg cells in the splenic and blood CD4$^+$T cells of control and CIA mice.

(C) The mRNA expression of Foxp3 in the spleen and ALNs of control and CIA mice.

Data were expressed as the mean ± SEM (n=6). * $p < 0.05$, **$p < 0.01$ compared with the control group by Student's t test.

Promotion of miR-143-3p on Treg cells differentiation in vitro

As previously mentioned, the expression of miR-143-3p has been associated with Treg cells differentiation in RA, which considered as a critical regulator of peripheral immune tolerance and homeostasis[6]. Hence, to directly investigate the potential effect of miR-143-3p on the differentiation of Treg cells, we cultured the naïve CD4$^+$T cells from C57BL/6 mice under induced Treg (iTreg) cell–polarizing conditions in vitro. As controls, naive CD4$^+$T cells were activated with anti-CD3ε/CD28 antibodies without the addition of differentiating cytokines (Th0 cells). We found that miR-143-3p mRNA expression was up-regulated in Treg cells differentiation when compared with Th0 (Figure 6A). Meanwhile, miR-143-3p shRNAs and mock shRNAs were infected into naïve CD4$^+$ T cells, and then cultured in the condition of Th17-polarization. The successful infection, which significantly altered the expression of the intracellular miR-143-3p, was verified by qPCR (Figure 6B). The overexpression of miR-143-3p markedly elevated the mRNA levels of forkhead box P3 (Foxp3), which was the master transcription factor of Treg cells. In contrast, expression of Foxp3 mRNA was notably reduced in the down-regulated cell population (Figure 6C). This funding indicates that miR-143-3p is capable to facilitate Treg cells differentiation in vitro.

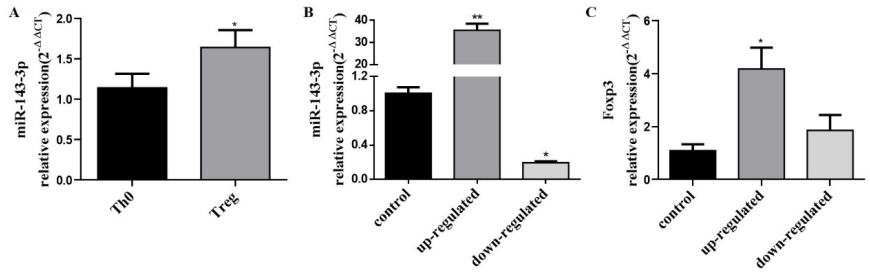

Figure 6. Promotion of miR-143-3p on Treg cell differentiation in vitro.

(A) The expression of miR-143-3p in CD4$^+$T cells (with the condition of Treg-polarization). Data were expressed as the mean ± SEM (n=3). * $p < 0.05$, **$p < 0.01$ compared with the control group by Student's t test.

(B) The expression of miR-143-3p in CD4$^+$T cells (with the condition of Treg-polarization) infected with miR-143-3p shRNAs and mock shRNAs. Data were expressed as the mean ± SEM (n=3). * $p < 0.05$, **$p < 0.01$, by one-way ANOVA with Dunnett's post-hoc test.

(C) The expression of Treg-related gene Foxp3 in the shRNA-infected cells. Data were expressed as the mean ± SEM (n=3). * $p < 0.05$, **$p < 0.01$, by one-way ANOVA with Dunnett's post-hoc test.

Overexpression miR-143-3p ameliorates joint injury and increases Treg cells proportion in CIA mice

Considering the positive role of miR-143-3p on Treg, we hypothesized that overexpressing miR-143-3p may ameliorate joint injury of CIA mice. We treat CIA mice with 2 mg/kg of miR-143-3p mimics at day 28

and day 35 tail vein injection after the first immunization and evaluated disease development. The high expression of miR-143-3p were observed, which verified the success transfection (Figure 7G). We found that miR-143-3p mimics markedly inhibited the development of CIA, with a significant reduction of arthritis score, paw thickness (Figure 7A-C) and histopathological scores (Figure 7D-F), when compared to mimics control group. Additionally, the production of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, remarkably decreased in the miR-143-3p mimic group (Figure 7H). Overall, our data indicate a global anti-inflammatory effect of miR-143-3p and confirm the potential of miR-143-3p as a novel therapeutic target for RA.

Next, the effect of miR-143-3p mimic on the response of Treg cells was examined. MiR-143-3p mimic treatment resulted in the increase of Treg cells than in mimics control group, which was in accord with the results in vitro (Figure 8A-B). As expected, the level of IL-10 and expression of Foxp3 mRNA were also found at a relatively higher level in miR-143-3p-treated mice (Figure 8C–D). In conclusion, these data demonstrate that miR-143-3p exerts a protective role in the progression of CIA through promoting Treg cells differentiation.

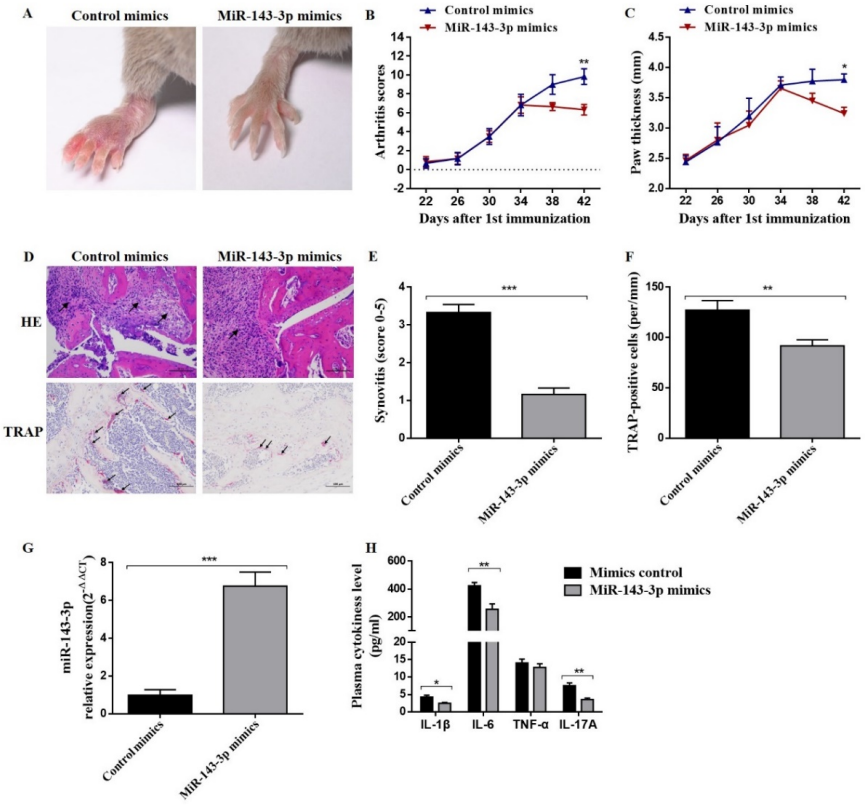

Figure 7. MiR-143-3p treatment inhibits inflammation and the progression of arthritis in CIA mice.

DBA/1J mice were immunized with bovine type II collagen emulsified in complete Freund adjuvant and incomplete Freund adjuvant. Mice were treated at day 28 and day 35 after the first immunization with saline (1 ml/kg) or miR-143-3p mimics negative control (2 mg/kg) or miR-143-3p mimics (2 mg/kg).

(A-C) Mean arthritis score and paw thickness in CIA mice treated with mimics control or miR-143-3p mimics. Data were expressed as the mean ± SEM (n=6). * $p < 0.05$, **$p < 0.01$, by one-way ANOVA with Dunnett's post-hoc test.

(D) Representative histopathological characteristics in the joints of miR-143-3p-treated CIA mice.

(E-F) Histological scores and the number of TRAP-positive cells in the joints of miR-143-3p-treated CIA mice. Data were expressed as the mean ± SEM (n=6). * $p < 0.05$, **$p < 0.01$ compared with the control mimics group by Student's t test.

(G) MiR-143-3p expression in CIA mice with miR-143-3p mimics or mimics control detected by qPCR analysis. Data were expressed as the mean ± SEM (n=6). * $p < 0.05$, **$p < 0.01$ compared with the control mimics group by Student's t test.

(H) The plasma levels of IL-1β, IL-6, TNF-α, IL-17A in CIA mice with miR-143-3p mimics or mimics control measured by ELISA. Data were expressed as the mean ± SEM (n=6). * $p < 0.05$, **$p < 0.01$ compared with the control group by Student's t test.

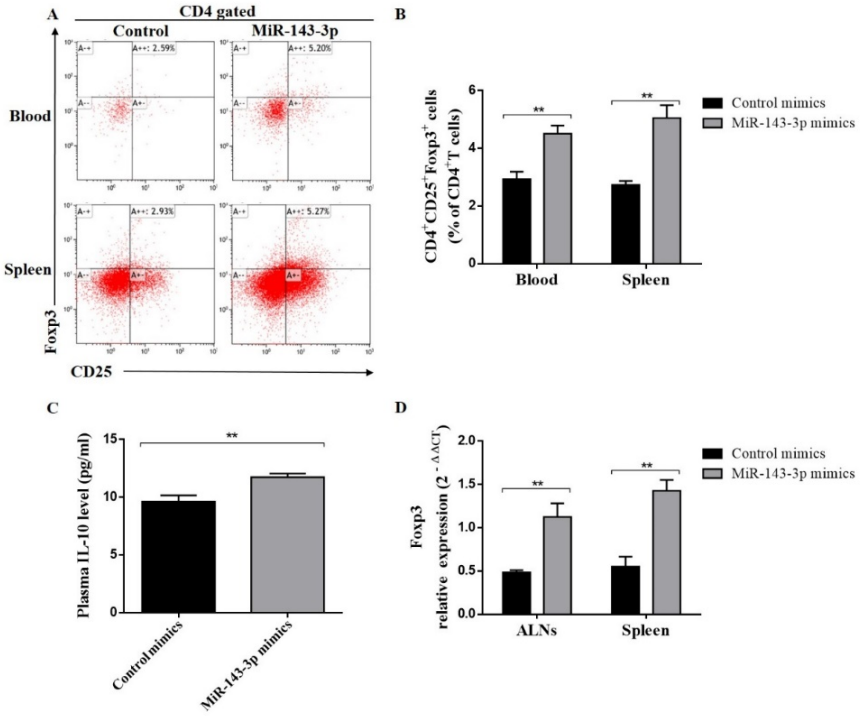

Figure 8. Induction of miR-143-3p overexpression facilitates Treg differentiation in CIA mice.

(A) Representative dot plots of $CD4^+CD25^+Foxp3^+$cells in blood and spleen of miR-143-3p-treated CIA mice.

(B) $CD4^+CD25^+Foxp3^+$cells in blood and spleen of miR-143-3p-treated CIA mice.

(C) The levels of IL-10 in the plasma of miR-143-3p-treated CIA mice.

(D) The mRNA expression of Foxp3 in the splenic and lymphatic $CD4^+$T cells of miR-143-3p-treated CIA mice.

Data were expressed as the mean ± SEM (n=6). * $p < 0.05$, **$p < 0.01$ compared with the control group by Student's t test.

Discussion

RA represents a chronic inflammatory autoimmune disease, which leads to irreversible destruction of the joints. The role of Treg cells in the pathogenesis is not entirely clear, as the decrease in the number of Treg cells in synovium and peripheral blood has been described in the past few years[33-35]. Our FCM data also demonstrate a significant decrease of Treg cells in PBMCs and spleen from CIA mice. The prevailing view in the functions of Treg cells is that they use different pathways to inhibit effector T cell proliferation and immune responses, including immunomodulatory cytokines such as IL-10, TGF-β and IL-35. For instance, IL-10 reporter (IL-10R) depletion results in the selective dysregulation of Th17 immune responses[36]. And several studies reported that mice with IL-10 deficient Treg cells could develop autoimmune diseases, such as spontaneous colitis[37]. Our data have validated the inverse correlation between plasma IL-10 and DAS28 scores in the RA patient cohort, suggesting that Treg-targeted might be an effective and potential strategy for RA treatment.

Previous studies have found that miRNAs play a key regulatory role in the pathogenesis of RA[38-40], and their regulatory role in Th cell differentiation and function has also been confirmed. Interestingly, results from emerging studies of miR-143-3p expression in RA patients appear to be conflicting, which has been reported to show different expression in different individuals with RA[17,27,28]. Hence, We conducted the clinical research and analysis of the correlation between miR-143-3p and Th in RA patients, and explored that miR-143-3p, with negatively correlated with DAS28, was actively associated with IL-10 secreted by Treg cells. The results in CIA mice were consistent with the clinical study. Meanwhile, a recent study reported that miR-143-3p was negatively associated with plasma inflammatory cytokines IL-6 in RA and sepsis[41,42], and IL-6 functions as a critical switch in inhibiting development of TGF-β-induced Treg cells[43]. Based on the above analyses, it is rational to hypothesize that miR-143-3p promoted the differentiation of Treg cells and treated Treg-deficiency diseases such as RA.

To further investigate the potential mechanisms associated with the protective effect of miR-143-3p in RA, we verified Treg cells as the critical node of miR-143-3p in this function. The elevated expression of miR-143-3p was verified to inhibit the mRNA levels of Foxp3 in vitro, which was the master transcription factor of Treg cells, indicating that miR-143-3p is capable to facilitate Treg cells differentiation. To further illuminate the role of miR-143-3p on the differentiation of Treg cells during the RA progress, the overexpression of miR-143-3p was conducted with synthetic miR-143-3p mimics in vivo. Our data corroborated that miR-143-3p mimic treatment resulted in a higher proportion of Treg cells in PBMC and spleen, along with the higher levels of IL-10 in plasma and Foxp3 mRNA in lymph nodes and spleen. These data demonstrate that miR-143-3p could upregulate Foxp3 expression to promote Treg cells differentiation.

Nevertheless, the molecular mechanism of the effect of miR-143-3p on the Treg cells differentiation is still unclear. In order to understand potential mechanism of miR-143-3p in Treg-deficiency of RA, we explored the microRNA database (miRBase at http://www.mirbase.org), which is the high confidence microRNA data set to identify potential target of the miR-143-3p[44,45]. With the TargetScan and miRDB miRNA target prediction tool, we have identified that Kras, c-maf, Mapk7, Bmp5, Etv6 may be the miR-143-3p targets. It is known that c-maf can affect the differentiation and function of $CD4^+$T cells through its unique regulatory mechanism, especially Treg cells[46]. It has been recent reported that c-maf was highly expressed in intestinal suppressive effector Treg cells (eTreg cells)[47], which were critical enforcers of the immune equilibrium. Moreover, Treg-derived IL-10 production was c-maf dependent[48]. These evidence indicate that the effect of miR-143-3p on Treg cells may be medicated by c-maf, which requires further studies for verification.

In conclusion, our results provide the evidence that the expression of miR-143-3p in PBMCs is negatively correlated with RA disease activity. And miR-143-3p could act as a key regulator of Treg cells differentiation to ameliorate CIA symptoms. These results provide a novel strategy to treat Treg-deficiency autoimmune diseases such as RA.

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Disclosure statement

The authors have no conflicts of interest to disclose.

Author Contributions

MY Shen and BP Jiang designed research, performed experiments, analyzed data, and wrote the paper; P Yang, MF Zhang, X Wang, H Zhu, ZN Gu and XP Zhou contributed significantly to the experiments and data analysis. LL Zhou and Y Lu designed the research and reviewed the manuscript. All authors reviewed and approved the final manuscript.

Data Available Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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