Original Article An analysis of Treg/Th17 cells imbalance associated microRNA networks regulated by moxibustion therapy on Zusanli (ST36) and Shenshu (BL23) in mice with collagen induced arthritis

Chuang Zhao^{1,2*}, Xiaoyan Li^{1,2*}, Yan Yang^{1,2}, Zunyuan Li^{1,2}, Miao Li³, Qian Tan⁴, Wei Liang^{1,2}, Zhidan Liu^{1,2}

¹Department of Acupuncture, Baoshan Branch of Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 201999, China; ²Department of Acupuncture, Baoshan Hospital of Integrated Traditional Chinese Medicine and Western Medicine, Shanghai 201999, China; ³Department of Geriatrics, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China; ⁴School of Basic Medical Science, Nanjing University of Chinese Medicine, Nanjing 210023, Jiangsu, China. ^{*}Equal contributors.

Received February 13, 2019; Accepted June 18, 2019; Epub July 15, 2019; Published July 30, 2019

Abstract: Moxibustion is an emerging alternative therapy for rheumatoid arthritis (RA) in the eastern world, especially in China. However, the mechanism underlying this condition has not yet been elucidated. This study aimed to explore how moxibustion reduced arthritis by regulating Treg/Th17 cell imbalance and the role of differentiationassociated keynote microRNAs (miRNAs). Moxibustion therapy was administered to mice with collagen-induced arthritis (CIA). The arthritis index, histopathological changes, inflammatory factors, and Treg/Th17 cell balance were analyzed. MiRNAs from CD4⁺ T cells were analyzed based on the RNA-seq technology. Treg/Th17 cell differentiation-related miRNAs and their target genes were identified from the online database of miRDB, TargetScan, and miRTarBase. The expression of miRNAs and their target genes was verified by quantitative reverse transcription polymerase chain reaction and Western blot analysis. The binding sites of miRNAs and target genes were predicted by miRDB, and the targeting relationship between them was verified by dual-luciferase reporter assay. Arthritis in mice with CIA was reduced by moxibustion therapy, and Treg/Th17 cell imbalance was regulated. Seventeen upregulated and twenty-three downregulated miRNAs were identified in moxibustion-treated mice. Seven upregulated miRNAs, seven downregulated miRNAs, and five Treg/Th17 cell differentiation-associated target genes were predicted. Among them, miR-144-3p and Hif1a were suggested to be the keynote miRNA and target gene, respectively, regulating the Treg/Th17 cell differentiation. In conclusions, moxibustion therapy plays a possible regulatory function in rebalancing Treg/Th17 cells by regulating miR-144-3p and its target gene *Hif1a* to treat CIA.

Keywords: Collagen-induced arthritis, microRNA, moxibustion, regulatory T cell, rheumatoid arthritis, T-helper cell 17

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation, vasculogenesis, and cartilage or bone destruction. This disorder is accompanied by pain and joint swelling or deformation that usually leads to loss of physical function and reduced quality of life [1]. Although the pathogenesis of RA is yet unclear, complex interactions between various functional immune cells and cytokine networks have been suggested [2, 3]. These networks are involved in the development of the disorder according to clinical evidence and experimental investigations.

The T-helper 17 (Th17) cells and regulatory T (Treg) cells are the two major T lymphocyte subsets with opposite functions and play a significant role in the progression of RA. Previous studies have shown that Th17 cells play a critical role in the onset of RA [4-6], while blocking Th17 cell differentiation exerts therapeutic benefits in collagen-induced arthritis (CIA), an experimental model of RA [7, 8]. In contrast to Th17 cells, CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells play a key role in reverse regulation, which is useful in preventing autoimmunity by suppressing the effector functions of the pathological T cells. A decline in the quantity and/or function impairment of Treg cells has been shown to aggravate various experimental autoimmune disorders, including CIA [9]. A shift in the balance between Th17 and Treg cells is regarded as a crucial mechanism for the development of RA [10]. Therefore, restoration of the balance in Treg/Th17 cells might exhibit a potential role in treating RA.

Moxibustion is a form of external therapy in traditional Chinese medicine, in which moxa is selectively burned as a medicinal material on acupoints or specific surficial regions to treat the disease. This method is similar to that of acupuncture but differs in manipulations. Moxibustion is widely used in treating RA in ancient China. Acupuncture has been proved to be effective in treating patients with RA and CIA [11-15]. Reportedly, moxibustion on BL23 (Shenshu) and ST36 (Zusanli) could reduce arthritis inflammation and improve symptoms [16, 17]. The mechanism of the disease might be related to the reduction in synovial hyperemia and edema, inflammatory cell infiltration, synovial cell proliferation, and tissue thickening [18], inducing synovial cell apoptosis [19, 20] and downregulating the expression of inflammation- or signaling pathway-related genes and proteins [21-28].

The imbalance between Th17 and Treg cells is crucial in the development of RA, which is regulated by microRNA (miRNA/miR), for example miR-155 [29, 30], miR-146a [30, 31], and miR-21 [32, 33]. Previous studies proved that moxibustion could treat RA by rebalancing Treg/ Th17 cells [34]. Moxibustion was shown to influence miR-155 and miR-146a, while miR-21 was not affected [35]. This indicated that miRNAs redeployed by moxibustion referred to a more complicated network and target genes.

To investigate this hypothesis, the present study examined the effects of moxibustion on Treg/Th17 rebalance, arthritis-related cytokines, and Treg/Th17 cell differentiation-related miRNAs and target genes in the presence of placebo cotton-based moxibustion in a mouse model of CIA.

Methods

Ethics statement

This study was approved by The Institutional Ethics Committee, Baoshan Hospital of Integrated Traditional Chinese Medicine and Western Medicine, Shanghai (approval no. 201412-11). All animal experiments were designed according to the principles of the Three Rs (replacement, reduction, and refinement) and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Animals

Thirty male DBA/1J mice (8-10 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China) and housed in a pathogen-free environment (n = 3 mice/ cage). The housing room was climate-controlled with a 12-h light-dark cycle, and the temperature and humidity were maintained at 25°C ± 2°C and 55% ± 5%, respectively. The animals were allowed access to regular, standard mouse chow and water ad libitum throughout the study.

Mice were randomly allocated to five groups (each group n = 6): normal, model (CIA), none treatment (CIA + none treatment), moxibustion (CIA + moxibustion treatment), and placebo (CIA + placebo treatment). The four CIA groups were mixed and injected with type II collagen to induce arthritis, then they were randomly allocated to four groups again after the establishment of the CIA model. The animals in the model group presented the onset state of CIA, and they were executed after the establishment of the CIA model. Mice in the no-treatment group presented the persistent state of CIA, which were executed along with the mice in the normal, moxibustion, and placebo groups.

Collagen injection followed by moxibustion was given in the moxibustion group, while the moxa was replaced by cotton in the placebo group and none treatment was given to the no-treatment group after CIA induction. Moxa or cottonbased moxibustion was applied on points BL23 (Shenshu) and ST36 (Zusanli) every alternate day for eight times. The arthritis score and paw volume (explained in the next section) for each mouse was determined on the first day and every alternate day after each treatment. Mice were sacrificed after 15 days, and the ankle joints were sampled for histopathological analysis. Spleen mononuclear cell (MNC) suspensions were acquired and examined for changes in the percentages of Treg and Th17 cells by fluorescence-activated cell sorting (FACS). In addition, quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) and Western blot analysis were applied to measure the mRNA and protein expression levels of Foxp3 and RORyt, respectively.

Induction and assessment of CIA

A CIA mouse model was established according to a previous study. Bovine type II collagen (Sigma, St. Louis, Missouri, USA) was solubilized in 0.1 mol/L acetic acid at a concentration of 2 mg/mL by stirring for 4 h at 4°C. To prepare Freund's complete adjuvant, 3-mg heat-inactivated Mycobacterium tuberculosis H37Ra (Difco, The Netherlands) was added to 1 mL of Freund's incomplete adjuvant (Sigma). Then, collagen was added dropwise to Freund's complete adjuvant (1:1 v/v), followed by emulsification with a homogenizer in an ice bath. A total of 25 male DBA/1J mice were immunized with an intradermal injection of the emulsion (50 µL) into the tail. A booster immunization was performed after 2 weeks of primary immunization, and the mice were evaluated for disease progression every alternate day. After 1 week of the first immunization, the mice were boosted intradermally with the same dose of collagen emulsion. The onset and progression of arthritis were monitored by recording the paw volume on alternate days. The severity of arthritis was scored by two independent observers in a blinded manner.

Each ankle was scored according to the severity of inflammation, where 0 = no evidence of redness or swelling, 1 = redness and slight swelling confined to the tarsal joints or ankle, 2 = redness and mild swelling extending from the ankle to the tarsal joints, 3 = redness and moderate swelling extending from the ankle to the metatarsal joints, and 4 = severe swelling encompassing the ankle, foot, and digits or ankylosis of the limb.

Moxibustion therapy

The moxibustion therapy was applied on the acupuncture points BL23 (Shenshu) and ST36

(Zusanli) after the CIA model was established successfully. BL23 (Shenshu) was localized on both sides of the spinous process of a second lumbar vertebra, while ST36 (Zusanli) localized on the anterolateral side of the knee, below the fibular head. The 45:1 purified moxa was purchased from Nanyang Aiyantang Moxa Co. (Henan, China) and reformed into a grain size, 1 mg each. The cotton used in the placebo group was also shaped identically. The grain-size moxa or cotton was burned at the acupoints for indirect moxibustion, slightly higher than the top of the animal hair to avoid direct moxibustion, as direct moxibustion would result in empyrosis if applied in mice.

Moxibustion was performed every alternate day for seven times, and six pieces of grainsized moxa were burned each time at every acupoint. The animals not receiving moxibustion therapy in the normal and no-treatment groups were maintained in the cages similar to that in moxibustion-stimulated animals. All mice within every group were executed by dislocation of the cervical spine on the second day from the end of last therapy.

Extraction of lymphocytes and CD4⁺ cells

After sacrifice, the mice were soaked in 75% ethanol for 10 min, and the spleens were harvested under sterile conditions. Subsequently, the spleen was cut into small pieces using oph-thalmic scissors, homogenized, and filtered using a 200-mesh steel sieve with the core of a 5-mL syringe to remove large pieces of the tissue. The homogenate was centrifuged at 400 g for 5 min at 4°C. The supernatant was discarded, and 5 mL of lysis buffer was added to the pellet. Then, the spleen cells were subsequent-ly washed once or twice with phosphate-buffered saline (PBS), and 1×10^6 cells/mL were subjected to flow cytometry in four tubes.

Hematoxylin and eosin staining

The freshly isolated ankle joint was washed with PBS, fixed with 10% formalin, and embedded in paraffin wax. After sectioning, 4-µm-thick sections were deparaffinized in xylene, followed by rehydration through an ethanol gradient. Subsequently, hematoxylin and eosin (HE) staining was performed prior to histopathological examination in a blinded manner.

FACS analysis of Treg and Th17 cells in splenocytes

Spleen MNC suspensions were passed through a nylon mesh, and erythrocytes were lysed osmotically. The remaining MNCs were resuspended and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and phycoerythrin (PE)-conjugated anti-Foxp3 (eBioscience, San Diego, California, USA). For intracellular interleukin (IL)-17 staining, the spleen MNCs were stimulated for 5 h with 20 mg/mL phorbol-12-myrisate-13-acetate, 50 mg/mL ionomycin, and 100 mg/mL monensin. After staining with FITC-anti-CD4, fixation, and permeabilization, the cells were stained with PE-conjugated anti-mouse/rat IL-17A (eBioscience). Subsequently, the percentages of CD4⁺ Foxp3⁺ Treg cells and CD4⁺ IL17⁺ Th17 cells were analyzed by flow cytometry (Beckman Coulter, Atlanta, Georgia, USA) using the Cell Quest software (Becton Dickinson, Lake Franklin, New Jersey, USA).

RNA isolation and real-time qPCR

The hind paws were collected and homogenized using a Tissue-Tearor. Total RNA was extracted using the Ultraspec Phenol Kit (Biotecx, Houston, Texas, USA) according to the manufacturer's protocols. Then, cDNA was synthesized from total RNA using the cDNA Synthesis Kit (Roche, Germany) and TagMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA). gRT-PCR detected the levels of Foxp3, RORyt, Hif1a, mTOR, smad2, smad3, STAT3, miR-708-3p, miR-30a-3p, miR-144-3p, miR-151-3p, miR-129-5p, miR-199a-5p, miR-106a-5p, miR-20a-5p, miR-322-5p, miR-20b-5p, and miR-340-5p using an SYBR-green detection system on an ABI-7500 Real-time PCR System (Applied Biosystems). The mRNA expression was expressed relative to glyceraldehyde-3-phosphate dehydrogenase as an internal control, while the miRNA expression was expressed relative to U6 as an internal control. The relative miRNA expression levels were evaluated using the $2^{-\Delta\Delta ct}$ method, and the expression levels were normalized relative to those of U6. The PCR amplification reaction was carried out in a 20-µL system, including 1 μ L of cDNA, 1 μ L of forward primer, and 1 μ L of reverse primer. The primer sequences used are listed in additional files (Tables S1 and S2). All PCR assays were performed in triplicate.

Enzyme-linked immunosorbent assay

The supernatant of treated cells was harvested to detect the concentrations of IL-1 β , IL-6, IL-10, IL-17, and TGF- β by enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's protocol of the ELISA kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to complete the experiment. Each sample was repeated three times, and the mean of values was considered for statistical analysis.

Western blot analysis

The expression of Foxp3 and RORyt were detected by Western blot analysis. The cell pellets were lysed in RIPA buffer (Beyotime Institute of Biotechnology, China) according to manufacturer's recommendations to the extract the total protein, which was estimated using the bicinchoninic acid kit (Beyotime Institute of Biotechnology, China). An equivalent of 20 µg/lane was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system and transferred to a polyvinylidene fluoride membrane (Millipore, Billica, Massachusetts, USA). Subsequently, the membrane was blocked with 5% bovine serum albumin (Hyclone, China) overnight at 4°C with gentle agitation, followed by probing with primary antibodies against Foxp3 and RORyt (Becton Dickinson) at room temperature for 2 h. The membrane was washed three times with TBST (BioTNT, China) and incubated with horseradish peroxidase-labeled secondary antibody (Becton Dickinson) at room temperature for 1 h. Finally, the membrane was developed using enhanced chemiluminescence (Thermo Fisher Scientific) for detecting immunoreactive bands. The images were acquired using the ChemiDoc MP System (Bio-Rad, Hercules, California, USA).

Microarray hybridizations

The mRNA expression was profiled using Agilent 4644 K mouse oligo arrays, and double-stranded cDNA was synthesized from 500 ng total RNA individually. An Agilent Linear Amplification Kit (Agilent Technologies, Santa Clara, California, USA) was used for cyanine 3-CTP-labeled complementary amplified RNA (cRNA), according to the manufacturer's recommendations. Cyanine-labeled complementary RNA was

hybridized to microarrays in SureHyb chambers (Agilent Technologies) using Agilent mouse 4 × 44 K oligonucleotide microarrays (Agilent Technologies) for 18 h at 60°C. Then, 44,000 oligonucleotide probes covering the entire mouse functional genome and internal control probes were included in each array. The arrays were washed and scanned according to the manufacturer's protocols.

MiRNA expression profiling using Agilent 8615 K mouse oligo arrays

Total RNA (100 ng), Cy-3-labeled using Agilent miRNA Complete Labeling and Hybridization Kit (Agilent Technologies), was dephosphorylated by incubation with calf-intestinal phosphatase at 37°C for 30 min, denatured in 100% Dimethyl sulfoxide (DMSO) at 100°C for 5 min, and labeled with perCp-Cy3 using T4 ligase at 16°C for 1 h. On an 8 × 15 K format Agilent mouse miRNA array slide, labeled RNA samples were hybridized to individual arrays, in which probes for 567 mouse miRNAs and 10 mouse gamma herpes virus miRNAs were included. Hybridizations were performed in SureHyb chambers (Agilent Technologies) for 20 h at 55°C, washed, and scanned according to the manufacturer's protocol.

Microarray data analysis

The oligo-mRNA and oligo-miRNA array slides were scanned using a DNA microarray scanner (Agilent Technologies), and the hybridization signals were extracted using the Agilent Feature Extraction software version 10.5. The gene expression profiles from CD4⁺ peripheral lymphocytes were analyzed by comparing the microarray hybridizations of the respective samples. The microarray numerical quantitative data, which was normalized to 75th percentile, were analyzed using the GeneSpring GX bioinformatics platform (http://www.agilent. com/chem/genespring) according to default instructions allowing for hierarchical clustering of samples of mice or genes based on an analysis of variance (ANOVA) statistical analysis ($P \leq$ 0.05), fold change \geq 2, and uncentered Pearson correlation metrics. The similarities and dissimilarities in gene expression were analyzed, and the functions of mRNAs were assessed by Gene Ontology (GO) annotations retrieved from the GeneSpring platform based on a corrected P value < 0.05 and cut-off = 0.1. Information about the miRNAs was retrieved from the miR-Base data bank (www.mirbase.org).

Cell transfection and luciferase reporter assay

MiR-144-3p mimics (5'-UACAGUAUAGAUGAUG-UACU-3'; Genepharma, China), negative control (5'-CAGUACUUUUGUGUAGUACAA-3'; Genepharma, China), and pGL3-Promoter (Promega, WI, USA) were used to transfect HEK293T cells and for transfection. miRDB was used to predict the targeted relationship between miRNAs and target genes, and 3'-UTR was their binding site. The wild- or mutant-type 3'-UTR of the target gene was inserted into the pGL3-Promoter and pRL-TK (Invitrogen, Carlsbad, California, USA) to transfect HEK293T cells. Then, the firefly luciferase reporter plasmids and miRNA mimics or negative control were used to transfect the HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific). The cells were grouped according to the difference in transfection type as follows: MT + mimics, MT + NC, WT + mimics, and WT + NC groups. The cells in each group were seeded into six-well plates at a density of 5×10^5 /mL, and 1 mL of cell suspension was added into each well. Then, these six-well plates were incubated in 5% CO₂, 37°C, and 95% humidity carbon dioxide incubator. These cells were collected and then incubated for 48 h, and luciferase activity was performed by dual-luciferase reporter assay system according to the manufacturer's protocol (Promega).

Statistical analysis

The SPSS 18.0 software (SPSS, Inc., Chicago, Illinois, USA) was used for the statistical analyses. Data were presented as the mean \pm standard deviation. Univariate ANOVA was used to analyze the differences among the groups. Nonparametric data were compared using the Dunnett's T3 test. A *P* value < 0.05 was considered statistically significant.

Results

Moxibustion therapy relieved symptoms in mice with CIA

After 10-20 days of first collagen immunization, redness and swelling in the joints appeared gradually, and all injected mice developed CIA within 7 days after second collagen immuniza-



Figure 1. Effects of moxibustion on foot swelling, arthritis index scores, and pathology of ankle articular surface in mice with CIA. A. Compared with the normal group, the joints showed obvious redness and swelling in mice with CIA. The redness and swelling subsided after moxibustion therapy, which was not obvious in the placebo group. B. Representative HE-stained sections (× 200) from the five groups demonstrated a massive accumulation of inflammatory cells in the swollen joints and apparent rough synovia in the ankle joint of the model, no-treatment, and placebo groups illustrating significant inflammation compared with the normal group, while the moxibustion group demonstrated less inflammatory cell infiltration and hyperplastic synovial cells in the ankle joint. C. Semi-Quantitative scoring of the inflammation revealed that moxibustion therapy significantly (P = 0.03) reduced the degree of joint inflammation, with a mean arthritis index score of 0.83 ± 0.98 versus 3.17 ± 0.98 in the no-treatment group and 3.10 ± 0.98 in the placebo group. D. Moreover, the moxibustion group achieved a significant decline (P < 0.01) with a low mean score of 1.17 ± 0.75 by HE staining versus 3.67 ± 0.52 in the model group, 3.17 ± 0.75 in the no-treatment group, and 3.00 ± 0.63 in the placebo group. Data are expressed as means \pm standard deviation. *P < 0.05 versus the normal group; $\mathbf{A} P < 0.05$ versus the model group.

tion. No adverse events were observed during the model establishment and interventions. All

six mice were included in each analysis in every group.



Figure 2. Levels of joint inflammation-associated cytokines in the spleen by moxibustion therapy. The levels of IL-1 β , IL-6, IL-17, IL-10, and TGF- β in the spleen were measured by ELISA after 15 days of the first intervention in mice with CIA. The mice with CIA were treated with moxibustion treatment (n = 6), cotton-based placebo treatment moxibustion (n = 6), or none treatment (n = 6). The levels of IL-1 β , IL-6, and IL-17 reduced (P = 0.00), while IL-10 and TGF- β were induced (P < 0.01) by moxibustion treatment compared with the model, no-treatment, and placebo groups. Data are represented as means ± standard deviation. *P < 0.05 versus the normal group; P < 0.05 versus the model group.

Figure 1A shows apparent redness and swelling in the joints in the model group compared with that in the normal group. The redness and swelling subsided after moxibustion therapy; however, it was not obvious in the placebo group. The histological analysis revealed no sign of inflammation in the ankle joint of the normal animals, but the ankle articular surface was rough, and the synovial layer was thinner in the CIA mice compared with the normal group.

Conversely, the articular surface was smooth, and the synovial layer became thicker after moxibustion compared with the no-treatment and placebo groups. The collagen-induced joint inflammation was considerably inhibited by moxibustion (Figure 1B). The recorded arthritis index score (Figure 1C) was analyzed by variance analysis of repeated measurement data, and the rank-sum test showed an obviously lower score in the moxibustion group than in the no-treatment and placebo groups. Moreover, the HE staining scores showed a difference among the four groups compared with the normal group, and a significant decline was observed in the moxibustion group compared with the model, no-treatment, and placebo groups (Figure 1D).

CIA in mice was reduced by moxibustion therapy

IL-1 β , IL-6, and IL-17 are proinflammatory cytokines; however, IL-10 and TGF- β are anti-inflammatory cytokines involved in the progression of CIA. ELISA (**Figure 2**) showed that the levels of IL-1 β , IL-6, and IL-17 in the spleen of mice increased significantly in the model, no-treatment, and placebo groups compared with the normal group. Mo-

xibustion treatment significantly decreased the levels of IL-1 β , IL-6, and IL-17 in the moxibustion group compared with the model group. No changes were observed in the no-treatment and placebo groups.

Treg/Th17 cells were rebalanced by moxibustion therapy in mice with CIA

Figure 3A shows the effect of moxibustion on the balance between Treg and Th17 cells in the



Figure 3. Treg/Th17 cells rebalanced by moxibustion therapy. A. Flow cytometry analysis revealed that the frequency of CD4⁺ FOXP3⁺ Treg cells was significantly lower and that of CD4⁺ IL17⁺ Th17 cells was significantly higher in the model group compared with the normal animals. Notably, moxibustion therapy restored the balance between Treg and Th17 cells, resulting in an elevated Treg cell frequency (P < 0.01) and reduced Th17 cell frequency (P < 0.01). No changes were observed in the no-treatment (P = 0.40) and placebo groups (P = 0.88) compared with the model group. B-D. qRT-PCR and Western blot analysis revealed that the mRNA abundance and protein expression of RORyt in the splenocytes in the model, no-treatment, and placebo groups were significantly greater compared with those in the normal group (P < 0.01). The moxibustion group showed a significant decrease in the RORyt levels compared with the aforementioned three groups at the end of the experiment (P < 0.01). Foxp3 mRNA abundance and protein expression were significantly lower in the model, no-treatment, and placebo groups compared with the normal group (P < 0.01). The moxibustion group showed a significant decrease in the RORyt levels compared with the aforementioned three groups at the end of the experiment (P < 0.01). The moxibustion group showed a significant decrease compared with the normal group (P < 0.01). The moxibustion group showed a significant decrease in the RORyt levels compared with the normal group (P < 0.01). The moxibustion group showed a significant decrease in the RORyt levels compared with the normal group (P < 0.01). The moxibustion group showed a significant decrease in the RORyt levels compared with the normal group (P < 0.01). The moxibustion group showed a significant increase compared with the aforementioned three groups at the end of the experiment (P < 0.01). Data are expressed as means ± standard deviation. *P < 0.05 versus the normal group.

CD4* splenocytes. The frequencies of CD4* FOXP3* Treg cells and CD4* IL17* Th17 cells

were measured by FACS. Moxibustion therapy reduced the number of Th17 cells while induc-

Moxibustion regulated Treg/Th17 cells associated microRNAs in CIA mice



Figure 4. Heatmap of miRNAs, GO, and KEGG enrichment. A and B. Heatmaps of miRNAs from CD4⁺ T cells expressed differently among the normal, CIA, and moxibustion groups. The colors from blue to red represent the higher to lower expression levels of miRNAs. Seventy-nine differentially expressed miRNAs were identified in CD4⁺ T cells from mice with CIA compared with the normal mice and ninety-two differentially expressed miRNAs in the moxibustion group compared with the CIA group. C. Annotated map of the KEGG pathway. The enrichment of differentially expressed miRNAs was concentrated on the Fanconi anemia pathway, cell apoptosis, and lysosomes. D. Annotated map of GO terms. The differential expression of miRNAs was focused on cellular metabolic processes, intracellular membraneenclosed organelle, and DNA-dependent ATPase activity. The x-axis indicates the count of genes in the function terms and pathways, and the y-axis indicates the GO terms and pathways.



Moxibustion regulated Treg/Th17 cells associated microRNAs in CIA mice

Figure 5. miRNA network and associated target genes affected by moxibustion therapy. A. The differentially expressed mRNAs and their target genes predicted by miRanda v3.3a. B. Seventeen upregulated mRNAs (mmu-miR-708-3p, mmu-miR-708-5p, mmu-miR-143-3p, mmu-miR-451a, mmu-miR-152-5p, mmu-miR-30c-2-3p, mmu-miR-30a-3p, mmu-miR-151-5p, mmu-miR-148a-3p, mmu-miR-144-3p, mmu-miR-486a-5p, mmu-miR-145a-5p, mmu-miR-486b-5p, mmu-miR-151-3p, mmu-miR-486a-3p, mmu-miR-6539, and mmu-miR-129-5p) and their target genes. C. Twenty-three downregulated mRNAs (mmu-miR-3963, mmu-miR-106a-5p, mmu-miR-200b-3p, mmu-miR-6690-3p, mmu-miR-19b-3p, mmu-miR-669a-3p, mmu-miR-20a-5p, mmu-miR-362-3p, mmu-miR-322-5p, mmu-miR-20b-5p, mmu-miR-19a-3p, mmu-miR-181a-1-3p, mmu-miR-194-5p, mmu-miR-141-3p, mmu-miR-150-5p, mmu-miR-20a-3p, mmu-miR-374b-5p, mmu-miR-339-5p, mmu-miR-363-3p, mmu-miR-340-5p, and mmu-miR-374b-5p, mmu-miR-340-5p, and mmu-miR-374b-5p, mmu-miR-340-5p, mmu-miR-363-3p, mmu-miR-340-5p, and mmu-miR-374b-5p, mmu-miR-340-5p, mmu-miR-363-3p, mmu-miR-340-5p, and mmu-miR-374b-5p, mmu-miR-340-5p, mmu-miR-363-3p, mmu-miR-340-5p, mmu-miR-340-5p,

	miRNAs	Log2-Fold Change	P.value.adj	Online data base		
UP/ DOWI				miRDB	TargetScan	miRTarBase
Up	mmu-miR-708-3p	-2.90237	0.00	-	Hif1a	-
Up	mmu-miR-30a-3p	-1.43885	0.00	-	Hif1a, Cul1	-
Up	mmu-miR-144-3p	-1.36164	0.00	Hif1a	Runx1	-
Up	mmu-miR-151-3p	-1.30657	0.00	Hif1a	Hif1a	-
Up	mmu-miR-486a-3p	-1.22408	0.00	-	Smurf1, Runx1, Cul1	-
Up	mmu-miR-129-5p	-1.05671	0.00	Runx1 Cul1	Runx1, Cul1	Hif1a
Up	mmu-miR-199a-5p	-1.02438	0.01	Hif1a	Rheb, Hif1a	Hif1a
Down	mmu-miR-106a-5p	1.706176088	0.00	Hif1a	-	Hif1a
Down	mmu-miR-20a-5p	1.298050129	0.00	Hif1a	-	-
Down	mmu-miR-322-5p	1.236220275	0.00	Smurf1	Hif1a	-
Down	mmu-miR-20b-5p	1.205675026	0.00	Hif1a	-	-
Down	mmu-miR-141-3p	1.139551352	0.00	Rheb	Runx1, Rheb	-
Down	mmu-miR-200a-3p	1.122867093	0.00	Rheb	-	-
Down	mmu-miR-340-5p	1.031262822	0.00	Runx1	Smurf1, Hif1a	-

Table 1. Selected up/down regulated miRNAs and their target genes predicted
--

ing Treg cells. qRT-PCR and Western blot analysis revealed that moxibustion therapy could downregulate the mRNA abundance and protein expression of RORγt and upregulate Foxp3 mRNA abundance and protein expression compared with the model, no-treatment, and placebo groups, (**Figure 3B-D**).

Moxibustion therapy affected CD4⁺ T cells through a complex miRNA network

Seventy-nine differentially expressed miRNAs were identified in CD4⁺ T cells from the mice with CIA compared with the normal mice, while 92 differentially expressed miRNAs were identified in the moxibustion group compared with the CIA group (**Figure 4A** and **4B**). Among the differentially expressed miRNAs in the moxibustion group, 17 miRNAs were upregulated (P < 0.05, fold change ≥ 2.0) and 23 were down-regulated by moxibustion (P < 0.05, fold change ≥ 2.0). Functional annotation of the differentially expressed mRNAs using Kyoto Encyclo-

pedia of Genes and Genomes (KEGG) enrichment analysis found them to be concentrated on Fanconi anemia pathway, cell apoptosis, and lysosomes (**Figure 4C**). GO (http://www. geneontology.org) identified them to be focused on cellular metabolic process, intracellular membrane-enclosed organelle, DNA-dependent ATPase activity, and so on (**Figure 4D**). The differentially expressed mRNAs and their target genes were predicted using miRanda v3.3a (**Figure 5**).

MiR-144-3p was associated with Treg/Th17 rebalance by moxibustion therapy through the target gene Hif1a

The 17 upregulated miRNAs and 23 downregulated miRNAs were used to select Treg/Th17 cell differentiation-related miRNAs. Among them, seven upregulated miRNAs, seven downregulated miRNAs, and five target Treg/Th17 cell differentiation-related genes (*Hif1a, Cul1, Runx1, Smurf1*, and *Rheb*) were identified



Figure 6. Selected miRNA rebalancing Treg/Th17 cells and potential target genes. The network of seven upregulated miRNAs (mmu-miR-708-3p, mmu-miR-30a-3p, mmu-miR-144-3p, mmu-miR-151-3p, mmu-miR-486a-3p, mmu-miR-129-5p, and mmu-miR-199a-5p), seven downregulated miRNAs (mmu-miR-106a-5p, mmu-miR-20a-5p, mmu-miR-322-5p, mmu-miR-20b-5p, mmu-miR-141-3p, mmu-miR-200a-3p, and mmu-miR-340-5p), and their five target Th17/Treg cell differentiation-related genes (*Hif1a, Cul1, Runx1, Smurf1*, and *Rheb*).

using the following three online databases: miRDB, TargetScan, and miRTarBase. *Hif1a* was considered to be the keynote target gene (**Table 1** and **Figure 6**). To verify the expression of the selected Treg/Th17 cell differentiationassociated miRNAs in mice, qRT-PCR was used. Only miR-144-3p was found to be downregulated in the CIA model group and then upregulated by moxibustion therapy (**Figure 7**). The expression of the selected Treg/Th17 cell differentiation-related genes (*Hif1a, RORyt, mT-OR, smad2, smad3, STAT3,* and *Foxp3*) was confirmed by qRT-PCR and Western blot analysis (**Figure 8**). Bioinformatics analysis by database miRDB revealed that *Hif1a* contained binding sequences complementary to the binding sites of miR-144-3p (**Figure 9A** and **9B**), and dual-luciferase reporter assay verified the targeting relationship between miR-144-3p and *Hif1a* (**Figure 9C**).

Discussion

RA is the most common autoimmune disease worldwide. Although decision-making support was not obtained from the systematic analysis [36-38], practice in history and several sporadic clinical studies in the present society provid-



Figure 7. Verification of moxibustion therapy–affected miRNAs by qRT-PCR. The selected *Hif1a*-targeted Th17/Treg cell differentiation-related miRNAs (analyzed from **Table 1**) in mice were verified by quantitative real-time PCR, and only miR-144-3p was found to be downregulated in the CIA model group and then upregulated by moxibustion therapy. **P* < 0.05 versus the normal group; **A***P* < 0.05 versus the model group.

ed the evidence for the applicability of acupuncture and moxibustion therapies for RA [11-13, 39]. Moreover, in experimental studies, acupuncture or electroacupuncture and moxibustion therapy proved to be efficient in treating animal models with CIA, which simulated the onset of RA on human beings. The underlying mechanism was related to regulation of inflammatory factors, immune cells, gene expression, and target proteins.

Among the immune cells, Th17 and Treg cells are the most interesting focus. Th17 and Treg cells were imbalanced in patients with RA or CIA animal model, and therapies that rebalanced Treg/Th17 cells were beneficial. Previous studies proved that moxibustion therapy could upregulate Foxp3 and downregulate *RORyt* gene [34], which were important Treg/Th17 cell differentiation-associated regulatory factors; however, how moxibustion therapy affected Treg and Th17 cell differentiation through mi-RNA, a significant part in the whole immunomodulatory network, was still unknown.

This study demonstrated that moxibustion therapy significantly reduced the severity of CIA in mice, which was substantiated by reduced arthritis index scores and HE staining scores in a previous study and confirmed again with a placebo treatment. The differentiation of imbalanced Treg/Th17 cells was rebalanced using moxibustion therapy, as Treg cells increased and Th17 cells decreased.

More significantly, to determine which miRNAs were in charge of the differentiation of Treg/

Th17 cells, this study established the upregulation and downregulation miRNA networks and identified the keynote miRNAs that affected the differentiation of Treg/Th17 cells. MiRNAs, such as miR-708-3p, miR-708-4p, miR-144-3p, and miR-3963, identified in this study were different from those in the study of T-cell posttranscriptional miRNAmRNA interaction network [40] and integrative computational Mrna-miRNA interaction analyses [41] in CIA. This indicated the involvement of a very complex re-

gulatory network, with different regulatory pathways working under different circumstances.

The present study identified miR-144-3p as the most critical miRNA associated with the rebalance of Treg/Th17 cells by moxibustion therapy, and database research analysis predicted that its target gene was hypoxia-inducible factor 1 α (*Hif1a*). HIF-1 is a nuclear protein with transcriptional activity and has a wide range of target gene profiles, including hypoxia adaptation, inflammation development, and tumor growth [42, 43]. *Hif1a* plays an essential role in the expression of Foxp3 and the suppressive functions of Tregs [44, 45]. Thus, it was hypothesized that moxibustion therapy rebalanced Treg/Th17 cell differentiation through *Hif1a*, which was regulated by miR-144-3p.

Hitherto, the CIA model is a commonly used animal model for studying RA [46, 47], which can be applied to mouse, rat, or rabbit. Although the underlying etiology and pathogenesis of RA have not yet been fully elucidated, the expression and balance of Treg/Th17 lymphocytes are under intensive research. Reportedly, the number of Th17 cells increased significantly, whereas the number of Treg cells decreased significantly in the peripheral blood of patients with RA compared with healthy controls [48]. These findings were also confirmed experimentally [8]. Interventions rebalancing the Treg/ Th17 lymphocytes might reduce the pathogenesis of patients with RA or CIA animal model [49-51]. Although both mouse and rat can be used, a mouse model was chosen in this study



Figure 8. Expression of potential target genes affected by moxibustion therapy. The expression of the selected Treg/ Th17 cell differentiation-related genes was confirmed by quantitative real-time PCR and Western blot analysis. The mRNA and protein expression levels of *Hif1a*, *RORyt*, *mTOR*, *smad2*, *smad3*, and *STAT3* reduced, but that of Foxp3 was induced by moxibustion therapy. *P < 0.05 versus the normal group; *P < 0.05 versus the model group.

to investigate the role of moxibustion in rebalancing Treg/Th17 cells and the miRNA network likely to be crucial. This is because it is easier to remove the hair to apply moxibustion therapy on the mouse, and the mouse model is comparable with previous studies on Treg/Th17 cell differentiation and the regulatory miRNA network.

The limitation of the present study was the comparability between animal studies and human clinical trials, as well as the potential difference between different acupoint selections among previous studies. Furthermore, the influence of different implementation methods of moxibustion therapy, such as treatment time, burning distance, and quantity of moxibustion materials, could not be distinguished in a single experiment. However, further studies are essential to verify the direct effect of miR-144-3p and *Hif1a* on Treg/Th17 cell differentiation *in vivo*.

Conclusions

In conclusion, the present study demonstrated that moxibustion therapy on ST36 and BL23 efficiently suppressed arthritis and reversed the imbalance of Treg/Th17 cells associated with arthritis in mice with CIA, thereby increasing the number of Treg cells and decreasing the number of Th17 cells. The underlying mechanism might be related to the regulation of miR-144-3p targeted with *Hif1a*. The present study of moxibustion therapy identified the potential therapeutic application and highlighted a potential mechanism underlying the intervention of moxibustion therapy in humans.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant number 81403465); Science Foundation of Bao-



A MicroRNA and Target Gene Description:

Figure 9. *Hif1a* is a direct target of miR-144-3p. A and B. Analysis by miRDB database revealed that *Hif1a* contained binding sequences complementary to the binding sites of miR-144-3p. C. Dual-luciferase reporter assay verified the targeting relationship between miR-144-3p and *Hif1a*. **P* < 0.05 versus NC-miR-144-3p.

shan Hospital of Integrated Traditional Chinese Medicine and Western Medicine, Shanghai (grant number GZRPYJJ-201706); Excellent Young Doctor Training Program from Baoshan District Health and Family Planning Commission, Shanghai (grant number bswsyq-2017-A02 and bswsyq-2017-A08); Young Doctor Talent Training Program from Baoshan Hospital of Integrated Traditional Chinese Medicine and Western Medicine, Shanghai (grant number 2016 and 2018).

Disclosure of conflict of interest

None.

Abbreviations

RA, rheumatoid arthritis; Th17 cells, T helper 17; Treg cells, regulatory T cells; CIA, collagen induced arthritis; miRNA/miR, microRNA; MNC, mononuclear cell; FACS, fluorescence-activated cell sorting; RT-qPCR, quantitative realtime polymerase chain reaction; HE, Hematoxylin and eosin; P-BS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; KEGG, kyoto encyclopedia of genes and genomes; GO, gene ontology.

Address correspondence to: Dr. Zhidan Liu, Department of Acupuncture, Baoshan Branch of Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, No. 181 Youyi Road, Baoshan District, Shanghai 201999, China. Tel: +86-21-56601100-237; +86-18916576769; E-mail: liuzhidan@vip.163.com

References

- Aletaha D, Smolen JS. Diagnosis and management of rheumatoid arthritis: a review. JAMA 2018; 320: 1360-1372.
- [2] Ridgley LA, Anderson AE, Pratt AG. What are the dominant cytokines in early rheumatoid arthritis? Curr Opin Rheumatol 2018; 30: 207-214.
- [3] Chen Z, Bozec A, Ramming A, Schett G. Anti-inflammatory and immune-regulatory cytokines in rheumatoid arthritis. Nat Rev Rheumatol 2019; 15: 9-17.
- [4] Niu Q, Cai B, Huang ZC, Shi YY, Wang LL. Disturbed Th17/Treg balance in patients with rheumatoid arthritis. Rheumatol Int 2012; 32: 2731-2736.
- [5] Ji L, Geng Y, Zhou W, Zhang Z. A study on relationship among apoptosis rates, number of peripheral T cell subtypes and disease activity in rheumatoid arthritis. Int J Rheum Dis 2016; 19: 167-171.
- [6] van Hamburg JP, Tas SW. Molecular mechanisms underpinning T helper 17 cell heterogeneity and functions in rheumatoid arthritis. J Autoimmun 2018; 87: 69-81.

- [7] Brand DD, Kang AH, Rosloniec EF. The mouse model of collagen-induced arthritis. Methods Mol Med 2004; 102: 295-312.
- [8] Hashimoto M. Th17 in animal models of rheumatoid arthritis. J Clin Med 2017; 6.
- [9] Yu Q, Xu M, Yu F, Jin Y. CD4⁺CD25⁺ regulatory T cells as a therapeutic target in rheumatoid arthritis. Cent Eur J Immunol 2014; 39: 100-103.
- [10] Lina C, Conghua W, Nan L, Ping Z. Combined treatment of etanercept and MTX reverses Th1Th2, Th17Treg imbalance in patients with rheumatoid arthritis. J Clin Immunol 2011; 31: 596-605.
- [11] Tam LS, Leung PC, Li TK, Zhang L, Li EK. Acupuncture in the treatment of rheumatoid arthritis: adouble-blind controlled pilot study. BMC Complement Altern Med 2007; 7: 35.
- [12] Shetty GB, Mooventhan A, Anagha N. Effect of electro-acupuncture, massage, mud, and sauna therapies in patient with rheumatoid arthritis. J Ayurveda Integr Med 2015; 6: 295-299.
- [13] Yim YK, Lee H, Hong KE, Kim YI, Lee BR, Son CG, Kim JE. Electro-acupuncture at acupoint ST36 reduces inflammation and regulates immune activity in collagen-induced arthritic mice. Evid Based Complement Alternat Med 2007; 4: 51-57.
- [14] Li J, Li J, Chen R, Cai G. Targeting NF- κ B and TNF- α activation by electroacupuncture to suppress collagen-induced rheumatoid arthritis in model rats. Altern Ther Health Med 2015; 21: 26-34.
- [15] Zhu J, Chen XY, Li LB, Yu XT, Zhou Y, Yang WJ, Liu Z, Zhao N, Fu C, Zhang SH, Chen YF. Electroacupuncture attenuates collagen-induced arthritis in rats through vasoactive intestinal peptide signalling-dependent re-establishment of the regulatory T cell/T-helper 17 cell balance. Acupunct Med 2015; 33: 305-311.
- [16] Hu L, Hao F, Zhong F, Qin LH, Xia XH, Chu HR, Luo L, Cai RL. Multicenter randomized controlled clinical study of rheumatoid arthritis with moxibustion. Glob Trad Chin Med 2011; 4: 401-405.
- [17] Hongwu Y, Yan Z, Yuzhen P, Jia Y, Bingkun W, Xue H, Yunyan C. Clinical efficacy of moxibustion supplemant on rheumatoid arthritis and the exploration on its mechanism. Zhongguo Zhen Jiu 2016; 36: 17-20.
- [18] Luo L, Hu L, He L, Tang ZL, Song XG, Dirckinck-Holmfeld L, Cai RL. Effect of moxibustion on ultrastructure of synovial cells in rheumatoid arthritis rats. Zhen Ci Yan Jiu 2011; 36: 105-109.
- [19] Tang ZL, Song XG, Wang NX, Wang YX, Zhang FQ, Hou ZM, Chen QZ, Xu B. Effect of moxibustion on synovial cell apoptosis and pathologi-

cal changes in adjuvant arthritis rats. J Trad Chin Med 2004; 22: 226-227.

- [20] Peng CY, Hu L, Wu ZJ, Cai RL, He L. Effect of moxibustion on apoptosis of synoviocytes in rheumatoid arthritis model rats. J Yunnan Colleg Trad Chin Med 2013; 36: 52-55.
- [21] Yao J, Hu, L, Song XG, Zheng BZ, Zhou F, Zhang C. Influence of moxibustion at "Shènshù" and "Zúsānlī" on Ras-MAPK signal pathways in synovial tissues of rats with experimental rheumatoid arthritis. W J Acup-Moxi 2013; 23: 29-33.
- [22] Liu XG, Huang DJ, Hao L, Song KY, Yu SG, Chen XL. Effect of moxibustion on hypothalamic-pituitary-adrenal axis in rats with rheumatoid arthritis. Chin J Basic Res Trad Chin Med 2003; 9: 57-59.
- [23] Gao J, Liu XG, Huang DJ, Tang Y, Zhou HY, Yin HY, Chen T. The role of HPAA in regulating NFkB signaling pathway in synovial tissue of rheumatoid arthritis rats by Moxibustion. Zhen Ci Yan Jiu 2010; 35: 198-203.
- [24] Zheng BZ, Hu L, Song XG, He L, Wu ZJ, Cai RL, Cai RL, Zhang C, Zhou F, Yao J. Effect of moxibustion on the expression of POMCmRNA and PDYNmRNA in the hypothalamus of rats with rheumatoid arthritis. Zhongguo Zhen Jiu 2013; 33: 433-437.
- [25] Ma WB, Liu XG, Zhou HY. Effects of chronological moxibustion on circadian rhythm activities of hypothalamus-pituitary-axis in rheumatoid arthritis rats. Zhen Ci Yan Jiu 2016; 41: 100-107.
- [26] Xu X, Wang MM, Sun ZL, Zhou DP, Wang L, Wang FQ, Xu ZY, Ma Q. Discovery of serum proteomic biomarkers for prediction of response to moxibustion treatment in rats with collageninduced arthritis: an exploratory analysis. Acupunct Med 2016; 34: 184-193.
- [27] Yin Y, Chen TW, Zhang R, Ma WZ. Effect of moxibustion on serum IL-17 and TNF- α levels in collagen-induced arthritis rats. Zhen Ci Yan Jiu 2017; 42: 149-152.
- [28] Cai GW, Li J, Li J. Effect of warm needle moxibustion intervention on knee-joint swelling and expression of synovial SIRT 1 and NF-κB in rats with rheumatoid arthritis. Zhen Ci Yan Jiu 2017; 42: 397-401.
- [29] Yao R, Ma YL, Liang W, Li HH, Ma ZJ, Yu X, Liao YH. MicroRNA-155 modulates Treg and Th17 cells differentiation and Th17 cell function by targeting SOCS1. PLoS One 2012; 7: e46082.
- [30] Zhou Q, Haupt S, Kreuzer JT, Hammitzsch A, Proft F, Neumann C, Leipe J, Witt M, Schulze-Koops H, Skapenko A. Decreased expression of miR-146a and miR-155 contributes to an abnormal Treg phenotype in patients with rheumatoid arthritis. Ann Rheum Dis 2015; 74: 1265-1274.

- [31] Niimoto T, Nakasa T, Ishikawa M, Okuhara A, Izumi B, Deie M, Suzuki O, Adachi N, Ochi M. MicroRNA-146a expresses in interleukin-17 producing T cells in rheumatoid arthritis patients. BMC Musculoskelet Disord 2010; 11: 209.
- [32] Dong L, Wang X, Tan J, Li H, Qian W, Chen J, Chen Q, Wang J, Xu W, Tao C, Wang S. Decreased expression of microRNA-21 correlates with the imbalance of Th17 and Treg cells in patients with rheumatoid arthritis. J Cell Mol Med 2014; 18: 2213-2224.
- [33] Jin S, Chen H, Li Y, Zhong H, Sun W, Wang J, Zhang T, Ma J, Yan S, Zhang J, Tian Q, Yang X, Wang J. Maresin 1 improves the Treg/Th17 imbalance in rheumatoid arthritis through miR-21. Ann Rheum Dis 2018; 77: 1644-1652.
- [34] Liu Z, Li X, Zhao C, Chen C, Li M, Tan Q, Zhang L, Liang W. Effects of moxibustion on Treg/ Th17 cell and its signal pathway in mice with rheumatoid arthritis. Zhongguo Zhen Jiu 2017; 37: 1083-1091.
- [35] Zhao C, Li X, Chen C, Li M, Liang W, Liu Z. Moxibustion for CIA mice and the expressions of miRNA-155, miRNA-146a and miRNA-21. Clin Acupunct 2017; 33: 60-64.
- [36] Wang C, de Pablo P, Chen X, Schmid C, McAlindon T. Acupuncture for pain relief in patients with rheumatoid arthritis: a systematic review. Arthritis Rheum 2008; 59: 1249-1256.
- [37] Lee MS, Shin BC, Ernst E. Acupuncture for rheumatoid arthritis: a systematic review. Rheumatology (Oxford) 2008; 47: 1747-1753.
- [38] Choi TY, Kim TH, Kang JW, Lee MS, Ernst E. Moxibustion for rheumatic conditions: a systematic review and meta-analysis. Clin Rheumatol 2011; 30: 937-945.
- [39] Amezaga UM, Suarez-Almazor ME. Acupuncture in the treatment of rheumatic diseases. Curr Rheumatol Rep 2012; 14: 589-597.
- [40] Donate PB, Fornari TA, Macedo C, Cunha TM, Nascimento DC, Sakamoto-Hojo ET, Donadi EA, Cunha FQ, Passos GA. T cell post-transcriptional miRNA-mRNA interaction networks identify targets associated with susceptibility/resistance to collagen-induced arthritis. PLoS One 2013; 8: e54803.
- [41] Honardoost MA, Naghavian R, Ahmadinejad F, Hosseini A, Ghaedi K. Integrative computational mRNA-miRNA interaction analyses of the autoimmune-deregulated miRNAs and wellknown Th17 differentiation regulators: an attempt to discover new potential miRNAs involved in Th17 differentiation. Gene 2015; 572: 153-162.

- [43] Hsu TS, Lai MZ. Hypoxia-inducible factor 1α plays a predominantly negative role in regulatory T cell functions. J Leukoc Biol 2018; 104: 911-918.
- [44] Clambey ET, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P, de Zoeten EF, Cambier JC, Stenmark KR, Colgan SP, Eltzschig HK. Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. Proc Natl Acad Sci U S A 2012; 109: E2784-2793.
- [45] Westendorf AM, Skibbe K, Adamczyk A, Buer J, Geffers R, Hansen W, Pastille E, Jendrossek V. Hypoxia enhances immunosuppression by inhibiting CD4⁺ effector T cell function and promoting treg activity. Cell Physiol Biochem 2017; 41: 1271-1284.
- [46] Bendele, A. Animal models of rheumatoid arthritis. J Musculoskelet Neuronal Interact 2001; 1: 377-385.
- [47] Li SM, Yu YH, Li L, Wang WF, Li DS. Treatment of CIA Mice with FGF21 Down-regulates TH17-IL-17 Axis. Inflammation 2016; 39: 309-319.
- [48] Niu Q, Cai B, Huang ZC, Shi YY, Wang LL. Disturbed Th17/Treg balance in patients with rheumatoid arthritis. Rheumatol Int 2012; 32: 2731-2736.
- [49] Pesce B, Soto L, Sabugo F, Wurmann P, Cuchacovich M, López MN, Sotelo PH, Molina MC, Aguillón JC, Catalán D. Effect of interleukin-6 receptor blockade on the balance between regulatory T cells and T helper type 17 cells in rheumatoid arthritis patients. Clin Exp Immunol 2013; 171: 237-242.
- [50] Kosmaczewska A, Ciszak L, Swierkot J, Szteblich A, Kosciow K, Frydecka I. Exogenous IL-2 controls the balance in Th1, Th17, and Treg cell distribution in patients with progressive rheumatoid arthritis treated with TNF-alpha inhibitors. Inflammation 2015; 38: 765-774.
- [51] Liu J, Hong X, Lin D, Luo X, Zhu M, Mo H. Artesunate influences Th17/Treg lymphocyte balance by modulating Treg apoptosis and Th17 proliferation in a murine model of rheumatoid arthritis. Exp Ther Med 2017; 13: 2267-2273.

Moxibustion regulated Treg/Th17 cells associated microRNAs in CIA mice

	1		1	
Genes	Primers	Sequences (5'-3')	Annealing temperature (°C)	Cycle number
Foxp3	Forward Reverse	ACCTATGCCACCCTTATCC GCTCCTCTTCTTGCGAAAC	55	40
RORγt	Forward Reverse	CCTGGATGTGTAAAGAATG TAGACTGCTAACGAATCTG	60	40
GAPDH	Forward Reverse	CTGCCCAGAACATCATCC CTCAGATGCCTGCTTCAC	60	40
Hif1a	Forward Reverse	CAAGAAACCACCCATGAC GGCTCATAACCCATCAAC	56	40
mTOR	Forward Reverse	GACCACTGTGCCAGAATCC GGACACCAGCCAATGTAGC	58	40
Smad2	Forward Reverse	TAGTTCCTGCCTTTGCTAAGAG GAGCCTTTGGATGCTGATAATG	60	40
Smad3	Forward Reverse	TTCATGGTCCTCTGTTATC TTTCTCCTACAGCATTCTC	60	40
STAT3	Forward Reverse	GACTCAAAGCCACCTCATTC GCCTTGCCTTCCTAAATACC	55	40

Table S1. RNA primer sequences and reaction conditions of qRT-PCR

Table S2. MicroRNA primer sequences and reaction conditions of qRT-PCR

Genes	Primers	Sequences (5'-3')	Annealing temperature (°C)	Cycle number
mmu-miR-708-3p	Sense Antisense	ACACTCCAGCTGGGCAACUAGACUGUGAGC CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTAGAA	60	40
mmu-miR-30a-3p	Sense Antisense	ACACTCCAGCTGGGCUUUCAGUCGGAUGUU CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCTGCA	60	40
mmu-miR-144-3p	Sense Antisense	ACACTCCAGCTGGGUACAGUAUAGAUGA CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGTACA	60	40
mmu-miR-151-3p	Sense Antisense	ACACTCCAGCTGGGCUAGACUGAGGCUCC CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCTCAA	60	40
mmu-miR-129-5p	Sense Antisense	ACACTCCAGCTGGGCUUUUUGCGGUCUGG CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCAAGC	60	40
mmu-miR-199a-5p	Sense Antisense	ACACTCCAGCTGGGCCCAGUGUUCAGACUAC CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAACAG	60	40
mmu-miR-106a-5p	Sense Antisense	ACACTCCAGCTGGGCAAAGUGCUAACAGUGC CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACCT	60	40
mmu-miR-20a-5p	Sense Antisense	ACACTCCAGCTGGGUAAAGUGCUUAUAGUGC CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACCT	60	40
mmu-miR-322-5p	Sense Antisense	ACACTCCAGCTGGGCAGCAGCAAUUCAUGU CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCCAAA	60	40
mmu-miR-20b-5p	Sense Antisense	ACACTCCAGCTGGGCAAAGUGCUCAUAGUGC CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACCT	60	40
mmu-miR-340-5p	Sense Antisense	ACACTCCAGCTGGG UUAUAAAGCAAUGAGA CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAATCAG	60	40
U6	Forward Reverse	CTCGCTTCGGCAGCACA AACGCTTCACGAATTTGCGT	60	40

All reverse TRP sequences are 5'-TGGTGTCGTGGAGTCG-3'.