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Arsenic trioxide improves Treg and Th17 balance by modulating STAT3 in treatment-naïve rheumatoid arthritis patients

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ABSTRACT

Objective: We aimed to investigate the immunologic mechanisms by which arsenic trioxide (As_2O_3) may inhibit T helper 17 (Th17) cell differentiation while promoting regulatory T (Treg) cell generation by modulating signal transducer and activator of transcription 3 (STAT3) in treatment-naïve rheumatoid arthritis (RA) patients.

Methods: Naïve $CD4^+$T cells isolated by fluorescence-activated cell sorting from treatment-naïve RA patients and healthy controls were used to investigate the effect of As_2O_3 on the process of polarization and the related cytokines. STAT3 transfection experiments were conducted with small interfering RNA (siRNA) and lentivirus STAT3 to verify the mechanism of As_2O_3 on Th17-Treg balance in vitro. A collagen-induced arthritis (CIA) model was used to detect the clinical scores, histopathological change, bone destruction, Th17-Treg proportion and joint tissue immunohistochemistry.

Results: We found that As_2O_3 prevented activated naïve $CD4^+$T-cells from differentiating into Th17 cells and reduced cytokine production by activated Th17 cells by downregulating their signature transcription factors, STAT3 and orphan nuclear receptors. Notably, As_2O_3 reduced Th17 cells frequency while increasing Treg cells frequency under specific polarizing conditions in treatment-naïve RA patients by transfecting siRNA STAT3 and lentivirus STAT3. Furthermore, we noticed that applying As_2O_3 in the CIA model attenuated the infiltration of joint inflammation and bone destruction, and significantly improved the imbalanced Treg-Th17 ratio.

Conclusions: These data indicate that As_2O_3 may be a potential immune modulator for treatment-naïve RA patients that helps to balance of Treg and Th17 cells through modulating STAT3.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease that is characterized by synovial inflammation, joint destruction and bone erosion [1,2]. Although the etiology and pathogenesis of RA is still largely unknown, previous studies have indicated that the imbalance of regulatory T (Treg) and T helper 17 (Th17) cells probably plays an essential role in the development and progression of RA [3]. Th17 cells mainly promote inflammation by producing interleukin (IL)-17 and thus lead to the occurrence of autoimmune diseases. IL-17 can induce the production of chemokine receptors (e.g. CXCR3 and CCR6), proinflammatory cytokines (e.g. IL-23, IL-6 and tumor necrosis factor α (TNF-α) and tissue-destructive enzymes, such as matrix metalloproteinase (MMP) 13, resulting in tissue invasion and destruction as well as damage to articular cartilage and bone [4,5]. Recent studies reported that Th17 cells play a critical role in RA pathogenesis and development [6,7]. Th17 cells can be identified on the basis of IL-17 production or on the basis of chemokine receptor expression. Specifically, the absence of the chemokine receptors CXCR3 and CCR6 is considered a surface marker for Th17 cells [8,9]. In contrast, Treg cells mediate the anti-inflammatory responses by producing IL-10 and transform growth factor (TGF) β and maintaining peripheral immunological tolerance [10]. In RA development, the percentage of Treg cells reduces and their function is suppressed [11]. Treg cells are characterized by the expression of forkhead box protein 3 (Foxp3), which inhibits the function of the characteristic transcription factor orphan nuclear receptor (RORγt) of Th17 cells [12].

Signal transducers and activators of transcription (STATs) are cytoplasmic transcription factors which are activated by tyrosine phosphorylation in response to interferons (IFN) and IL-6 cytokines acting via the gp130 receptor [13]. STAT3 is associated with human malignancies [14], oncogenic transformation [15], angiogenesis [16] and

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invasion by tumor metastasis [17]. In RA synovial tissues, the expression of STAT3, which is a key transcription factor for Th17 cells. Knocking down of STAT3 by transfecting small interfering RNA (siRNA) into $CD4^+$T cells, alleviated the synovitis and infiltration of inflammatory cytokines, as well as increasing the proportion of Treg cells and the function of the transcription factor STAT5 of Treg cells [18,19]. RA synovial fibroblast cells transfected with hypoxia-induced factor 1 α (HIF1α) siRNA inhibited hypoxia-induced p-STAT3 detection; STAT3-siRNA also inhibited hypoxia-induced HIF1α [20]. Activation of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), a key negative regulator of human and mouse Th17 cells differentiation, selectively suppressed Th17 cell differentiation by inhibiting RORγt expression in $CD4^+$T cells [21]. In addition, PPARγ also downregulates the catabolic expression of matrix metalloproteinase 13 and increase Type II collagen α1 chain (Col2A1) gene expression [22].

In recent studies, As_2O_3 has received considerable attention mainly focusing on its ability to treat hematopoietic malignancy and inhibit STAT3 activity in gastric cancer cells [23]. Moreover, our previous studies have shown that As_2O_3 significantly suppresses angiogenesis and induces fibroblast-like synoviocyte apoptosis in a collagen-induced arthritis (CIA) model [24,25]. However, the extract mechanisms by which As_2O_3 has its anti-rheumatic effects and whether this occurs via modulation of the immune system are still unknown. In this study, we demonstrated the effect of As_2O_3 on treatment-naïve RA patients and the Treg-Th17 cell balance, and the involvement of STAT3 in this process. To our knowledge, however, there are no published studies of its therapeutic potential in RA. We conducted this study to examine the effects of As_2O_3 in improving Treg-Th17 balance and preventing inflammation and bone destruction with the use of RA $CD4^+$T subtype cells and an experimental animal model of RA.

2. Materials and methods

2.1. Subjects

The study group comprised 15 healthy female volunteers (aged 45.1 ± 12.3 years, mean ± SD) and 24 treatment-naïve patients with RA (20 female and 4 males, aged 51.42 ± 12.05 years, mean ± SD) who fulfilled the American College of Rheumatology 1987 revised criteria for RA [26] (Table 1). Treatment-naïve RA patients were recruited from the Department of Rheumatology in the first affiliated hospital of Harbin Medical University. Some patients were taking nonsteroidal anti-inflammatory drugs and none of the patients had received disease-modifying antirheumatic drugs or corticosteroids. The study was conducted with formal approval from the Ethical Committee of Harbin Medical University.

Table 1
Clinical and laboratory characteristics of the 24 patients with RA.[a]

Morning stiffness	18 (75)
Arthritis of ≥3 joints	21 (88)
Arthritis of hand joints	24 (100)
Symmetric arthritis	22 (92)
Rheumatoid nodules	1 (4)
Serum rheumatoid factor	21 (86)
Serum anti-CCP	19 (79)
Bone erosions	2 (8)
DAS28, mean ± S.E.M (range)	5.7 ± 0.3 (4.0–8.8)
Swollen joint count, mean ± S.E.M (range)	11.1 ± 1.9 (2–4)
Tender joint count, mean ± S.E.M (range)	10.8 ± 1.7 (3–28)
Disease duration (month), mean ± S.E.M (range)	6.4 ± 0.8 (1−12)

[a] Patients were diagnosed as having rheumatoid arthritis (RA) if they met ≥4 of criteria listed. Except where indicated otherwise, values are the number (%) of patients. Anti-CCP = anti-cyclic citrullinated peptide; DAS28 = disease activity score in 28 joints.

2.2. Peripheral blood cell isolation and purification of naïve $CD4^+$T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers and treatment-naïve RA patients, then separated by density gradient on Ficoll-Hypaque (MD Pacific Biotechnology, Tianjin, China). Naïve $CD4^+$T lymphocytes were purified on a high-speed cell sorter system (Moflo XDP, Beckman Coulter, USA). The obtained naïve $CD4^+$T cells had a purity of > 95%, which was confirmed by flow cytometry (FC500, Beckman Coulter, Indianapolis, IN, USA).

2.3. Flow cytometry

For intracellular cytokine detection, cells were stimulated with the corresponding Cell Activation Cocktail (with Brefeldin A) (Biolegend, San Diego, CA, USA) for 6 h. After surface staining for 15 min, the cells were resuspended in a fixation buffer, washed three times with a permeabilization solution (Biolegend, San Diego, CA) for 5 mins each at 1500 rpm. Intracellular cytokine staining was performed according to the manufacturer's protocol. The following reagents were used for human experiments: fluorescein isothiocyanate-conjugated CD4 (clone: 13B8.2), biotinylated and phycoerythrin-conjugated CD25 (clone: B1.49.9), peridinin chlorophyll A protein-Cy5-conjugated CD127 (clone: R34.34), brilliant violet 421-conjugated Foxp3 (clone: 206D), allophycocyanin-conjugated CCR6 (clone: G034E3), phycoerythrin-conjugated CXCR3 (clone: G025H7) and phycoerythrin-conjugated IL-17A (clone: SCPL1362). All these antibodies were purchased from Beckman Coulter (San Diego, CA, USA). The following reagents were used for mouse assays: anti-CD4-FITC (clone: RM4-5), anti-CD25-phycoerythrin-Cy5 (clone: PC61), anti-Foxp3- phycoerythrin (clone: MF-14), anti-CD4-phycoerythrin-Cy5 (clone: RM4-5), anti-CD3-FITC (clone: 17A2), anti-17A- phycoerythrin (clone: TC11-18H10.1), anti-IFN-γ-phycoerythrin (clone: XMG1.2) and anti-IL-4-PC7 (clone: 11B11). All these antibodies were purchased from Biolegend (San Diego, CA).

2.4. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

The methods used for RNA extraction and complementary DNA (cDNA) synthesis have been described previously [24]. mRNA expression was detected by using the SYBR Master Mix PCR kit (Bio-Rad, CA, USA). Transcripts were quantified by real-time quantitative polymerase chain reaction on an ABI PRISM 7500 sequence detector (Applied Biosystems, Foster City, CA, USA). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *β-actin*, and were calculated via the $2^{-\Delta\Delta Ct}$ method. All primer sequences are given in Table 2.

2.5. Cytokine quantification

For human in vitro assays, the cytokines IL-17A (D1700), IL-10 (D1000) and TGF-β1 (DB100B) purchased from R&D Systems (Minneapolis, USA), and human IL-6 (E-EL-H0102c), TNF-α (E-EL-H0109c), MMP13 (E-EL-H0134c), and IL-23 (E-EL-H0107c) were determined via differentiation with enzyme-linked immunosorbent assay (ELISA). In animal experiments, the cytokines in serum were also measured by ELISA: mouse IL-17A (E-EL-M0047c), mouse IL-10 (E-EL-M0046c), mouse TGF-β1(E-EL-M0051c) and mouse IL-6 (E-EL-M0044c) (all these reagents were purchased from Elabscience Biotechnology, Wuhan, China). The ELISA was performed according to the manufacturer's instructions.

2.6. Western blotting

Cells were lysed in a lysis buffer and the protein content of the lysates was determined with a bicinchoninic acid protein assay kit (Catalog no. P0011, Beyotime, Shanghai, China). The proteins including STAT3 (Catalog no. ab68153, Abcam, Cambridge, MA, USA), p-

Table 2
The primers as following.

Human Foxp3	Sense 5′-GGGTAGCCATGGAAACAGCA-3′
	Antisense 5′-TCGCATGTTGTGGAATTGAAGTAG-3′
Human IL-17A	Sense 5′-CTCTGTGATCTGGGAGGCAAA-3′
	Antisense 5′-CTCTTGCTGGATGGGACA-3′
Human STAT3	Sense 5′-GGAGAAACAGGATGGCCCAA
	Antisense 5′-ATCCAAGGGGCCAGAAACTG 3′
Human RORγt	Sense 5′-CGCCTACAATGCTGACA-3′
	Antisense 5′-ACCGTATTTGCCTTCAA-3′
Human MMP13	Sense 5′-CGTATTGTTCGCGTCATGCC-3′
	Antisense 5′-GTTCCAGCCACGCATAGTCAT-3′
Human Col2A1	Sense 5′-CATGGAGACTGGCGAGACTTG-3′
	Antisense 5′-GTGGACAGCAGGCGTAGGAA-3′
Human GAPDH	Sense 5′-GCACCGTCAAGGCTGAGAAC-3′
	Antisense 5′-TGGTGAAGACGCCAGTGGA-3′
Mouse Foxp3	Sense 5′-GGCCCTTCTCCAGGACAGA-3′
	Antisense 5′-GCTGATCATGGCTGGGTTGT-3′
Mouse IL-17A	Sense 5′-CCTCAAAGCTCAGCGTGTCC-3′
	Antisense 5′-GAGCTCACTTTTGCGCCAAG-3′
Mouse STAT3	Sense 5′-GACCCGCCAACAAATTAA-3′
	Antisense 5′-TCGTGGTAAACTGGACACCA-3′
Mouse RORγt	Sense 5′-TGTCCTGGGCTACCCTACTG-3′
	Antisense 5′-GTGCAGGAGTAGGCCACATT-3′
Mouse β-actin	Sense 5′-TGTCCTGGGCTACCCTACTG-3′
	Antisense 5′-GTGCAGGAGTAGGCCACATT-3

STAT3 (Catalog No. ab76315, Abcam, Cambridge, MA, USA) and STAT5 (Catalog no. ab32364, Abcam, Cambridge, MA, USA) were determined by western blot analysis as described previously [22].

2.7. RNAi gene-silencing studies

STAT3-knockdown experiments were performed by transfecting specific STAT3 siRNA (GenePharma, Shanghai, China) into $CD4^+$T cells from RA peripheral blood. The $CD4^+$T cells were plated in six-well plates at a density of 5×10^5 cells per well with antibiotic-free RPMI 1640 supplemented with 10% FBS and grown to 50–70% confluence and transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The final concentration of the siRNA duplex was 100 pmol. The siRNA sequences targeting STAT3 were: (forward)_5′-CCACUUUGGUGUUUCA UAATTGAC-3′ and (reverse) 5′-UUAUGAAACACCAAAGUGGTT-3′; these sequences and a scrambled control (a nonsense siRNA of the target sequence) were purchased from Gene Pharma. The cells were then harvested and activated by adding anti-CD3 (2 μg/mL) and anti-CD28 (4 μg/mL) (Biolegend, San Diego, CA, USA). IL-1 β (10 ng/mL), IL-6 (20 ng/mL), TGF-β (1 ng/mL) (Biolegend, San Diego, CA, USA), IL-23 (100 ng/mL) (R&D Systems, Minneapolis, MN, USA) were added for Th17 cells polarization, and TGF-β (2 ng/mL) and IL-2 (20 U/mL) (Biolegend, San Diego, CA, USA) were added for Treg cells polarization. At the same time, As_2O_3 (0.5 μM) (Yitaida Pharmaceutical Factory, Harbin, Heilongjiang, China) was added once a day for 3 days.

2.8. Lentivirus vector transfection assay

The GV358 lentivirus vector (Genechem, Shanghai, China) were used to upregulate STAT3 expression in naïve $CD4^+$T-cell. The assays of lentivirus infection assays were performed according to the manufacturer's instructions. Briefly, 50 μL of the GV358 lentivirus vector and 50 μL of polybrene (50 μg/mL; Genechem, Shanghai, China) were added in a 24-well plate containing 500 μL RPMI 1640 and 1×10^5 naïve $CD4^+$T-cells. After 8 h, the transfection medium was replaced by 500 μL RPMI 1640 containing 10% fetal bovine serum. The cells were then harvested and activated by adding anti-CD3 (2 μg/mL) and anti-CD28 (4 μg/mL).

2.9. The CIA model, clinical evaluation and assessment of arthritis severity

Male DBA/1 J mice (5–8 weeks old and 20 g ± 2 g in weight, Charles River, Beijing, China) were housed under specific pathogen-free (SPF) conditions at the veterinary institute of Harbin Medical university and were fed with standard mouse chow and water. All experimental procedures were examined and approved by the Institutional Animal Care and Use Committee of Harbin Medical University. The method for the CIA mouse model was performed in our laboratory as described previously [24]. The scoring system was used according to a protocol described previously [27]. The mean body-weight of each group was calculated and used as a parameter of toxicity (see in Data in brief) [28] as described previously [29].

2.10. Micro-computed tomography

A Quantum GX Imaging System (PerkinElmer, Inc., Hopkinton, MA, USA) was used to evaluate structural changes in the hind paws of the CIA mice. The hind paws (from the tip of the toes to the middle of the tibia) obtained from experimental mice were scanned and reconstructed into a three-dimensional structure. Joints destruction was scored in a blinded manner on a 0 to 4 scale, ranging from no damage to severe joint damage. Mean CT values of hind paws were calculated with Caliper Analyze software to assess bone loss.

2.11. Histological analysis

Joint tissue specimens from mice with CIA were fixed 4% formalin, decalcified for 3 weeks in 10% EDTA, dehydrated, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin. The mouse joint sections were scored for changes in synovial proliferation, inflammation, cartilage damage and bone erosion, and histological scores were assessed as previously described [24].

2.12. Immunohistochemistry analysis

The immunohistochemical analysis was performed as previously described with some modifications. Mouse joint tissues and spleen sections (5 μm) were incubated with primary Abs against STAT3 (BS-20382R, Bioss, Beijing, China), IL-17A (DF6127, Affbiotech, USA), Foxp3 (BA2032-1, Boster, Wuhan, China), IL-10 (BS-20373R, Bioss, Beijing, China) for 2 h. Then the sections were incubated using polymer HRP detection system (PV6001, ZSGB-BIO, Beijing, China) for 30 min. The final colored product was developed using DAB substrate Color Kit (ZSGB-BIO, Beijing, China). Finally, the sections were counterstained with hematoxylin and photographed using a microscope (Leica, Mannheim, Germany) in three randomly selected areas at a magnification of 400×. The positive stained cells and total were counted by different three observers, and the means of the ratio of these two groups were calculated by Image Pro Plus software.

2.13. Statistical analysis

Statistical significance was determined with GraphPad Prism Software (Version 6 for Windows; Graphpad Prism, San Diego, CA, USA). Simple comparisons were made via the unpaired two-tailed Students's *t*-test for parametric data or the Mann-Whitney test for non-parametric data, as appropriate. Multigroup comparisons of the means were carried out by one-way analysis of variance with post hoc contrasts by Turkey's test. *p*-Values of 0.05 or less were considered to be statistically significant. All data are presented as means ± standard errors of the mean.

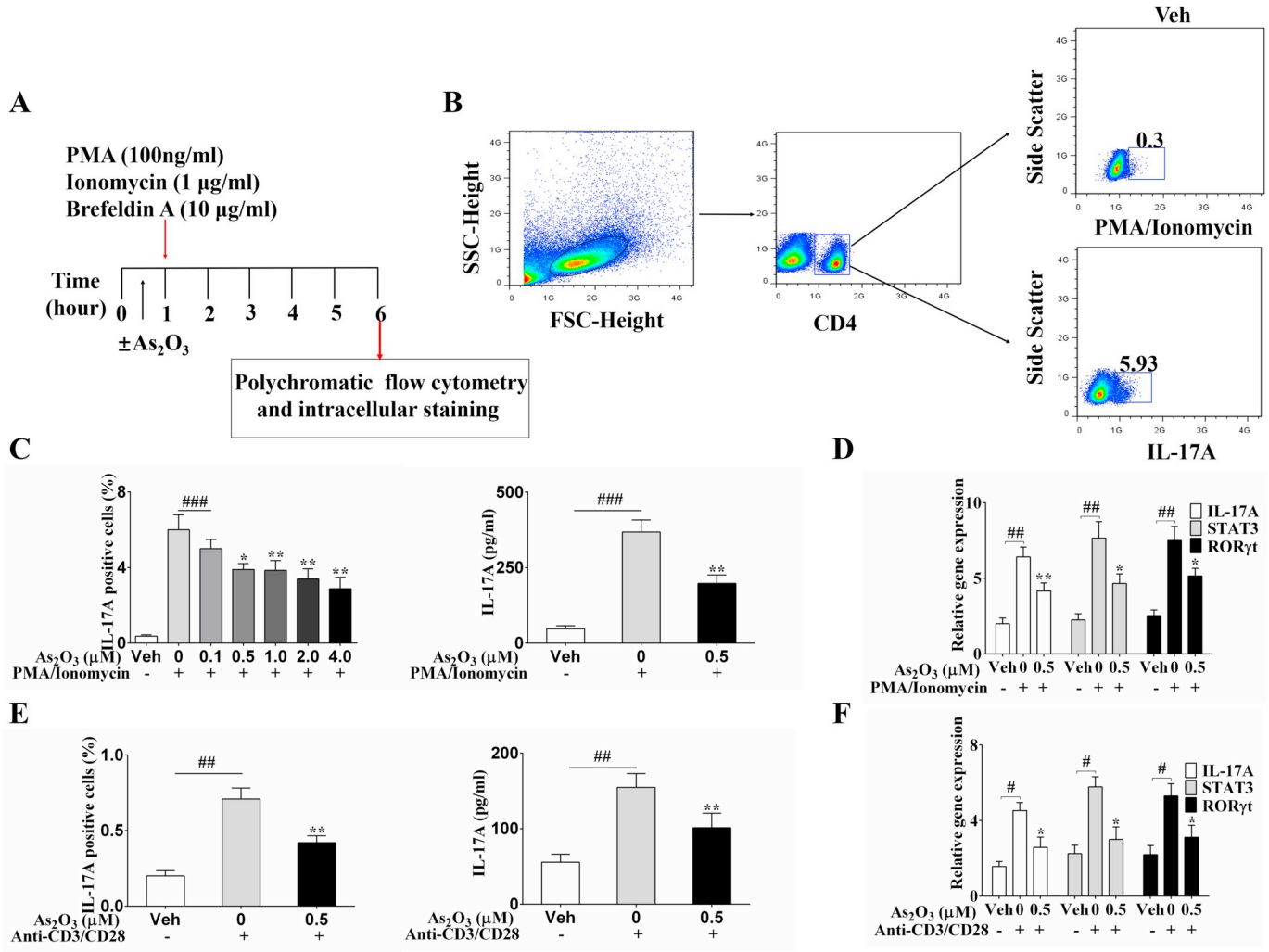

Fig. 1. As_2O_3 dose-dependently reduce IL-17A production and STAT3 expression from $CD4^+$T cells. (A) Peripheral blood mononuclear cell (PBMCs) (1×10^6 cells per well) were left untreated (Veh) or treated with different concentrations (0.1 to 4.0 μM) of As_2O_3 for 30 min. Cells were then stimulated with PMA/Ionomycin for 6 h, stained at the cell surface and intracellularly, and analyzed by flow cytometry. (B) Representative cytofluorimetric plot of the gating strategy for IL-17A evaluation in $CD4^+$T cells from treatment-naïve RA patients. PBMCs were appropriately gated according to physical parameters. (C to F) Percentages, protein and mRNA levels of intracellular IL-17A, signal transducer and activator of transcription 3 (STAT3) and transcription factor orphan nuclear receptor (RORγt) of Th17 cell from $CD4^+$T cells stimulated with PMA/ionomycin [(C) and (D) or with anti-CD3/CD28 (E) and (F)]. Data are means ± SEM of six independent experiments. # $p < 0.05$, ## $p < 0.01$ vs nonpolarized $CD4^+$T cells; * $p < 0.05$, ** $p < 0.01$ vs $CD4^+$T cell nontreated with As_2O_3 (one-way ANOVA).

3. Results

3.1. As_2O_3 modulate Th17 cells responses in $CD4^+$T cells from treatment-naïve RA patients

To investigate the effects of As_2O_3 on $CD4^+$T cell responses, we performed initial experiments to assess whether increasing the concentrations of As_2O_3 (from 0.1 to 4.0 μM) could affect the production of specific cytokines that characterize the main proinflammatory Th17 cells, namely IL-17A from human $CD4^+$T lymphocytes (Fig. 1A). When activated with phorbol 12-myristate 13-acetate (PMA)/ionomycin $CD4^+$T cells produced large amounts of intracellular IL-17A (Fig. 1B, C), which was reduced upon pretreatment with As_2O_3 (Fig. 1C). As_2O_3 suppressed IL-17A production in a dose-dependent manner and substantially reduced cytokine production at doses as low as 0.5 μM. The lowest concentration (0.1 μM) of As_2O_3 only showed a slight and non-significant reduction in IL-17A production from naïve $CD4^+$T cells (Fig. 1C). For this reason, in our further experiments, As_2O_3 was used at the lowest effective concentration (0.5 μM). The initial results showed that As_2O_3 might indeed be effective in modulating $CD4^+$T cells from treatment-naïve RA patients.

We next tested the possible impact of As_2O_3 on the mRNA expression of STAT3, RORγt and cytokine IL-17A from Th17 cells from PMA/ionomycin-activated $CD4^+$T cells. STAT3, RORγt and IL-17A also decreased significantly in the presence of As_2O_3 treatment (Fig. 1D). Because the cytokine profile of human $CD4^+$T cells may be differently determined, depending on the experimental conditions, we also investigated the immunomodulatory role of As_2O_3 by using a specific and physiological stimulus to activate $CD4^+$T cells with anti-CD3/CD28. Cytokine production and mRNA expression levels after stimulation of $CD4^+$T cells with anti-CD3/CD28 was almost identical to that of PMA/ionomycin stimulation, though the expression levels of the intracellular cytokines IL-17A, transcription factor STAT3 and RORγt expression were lower, as expected (Fig. 1E, F). Overall, As_2O_3 might regulate Th17 cells response in treatment-naïve RA patients.

3.2. As_2O_3 increased Treg cell-related signal cytokines and decreased Th17 cell-related signal cytokines from treatment-naïve RA patients

In humans, different lineages of the T helper cells are characterized by differential expression of the chemokine receptors participating in cell homing to lymphoid and peripheral tissues. Specifically, classical

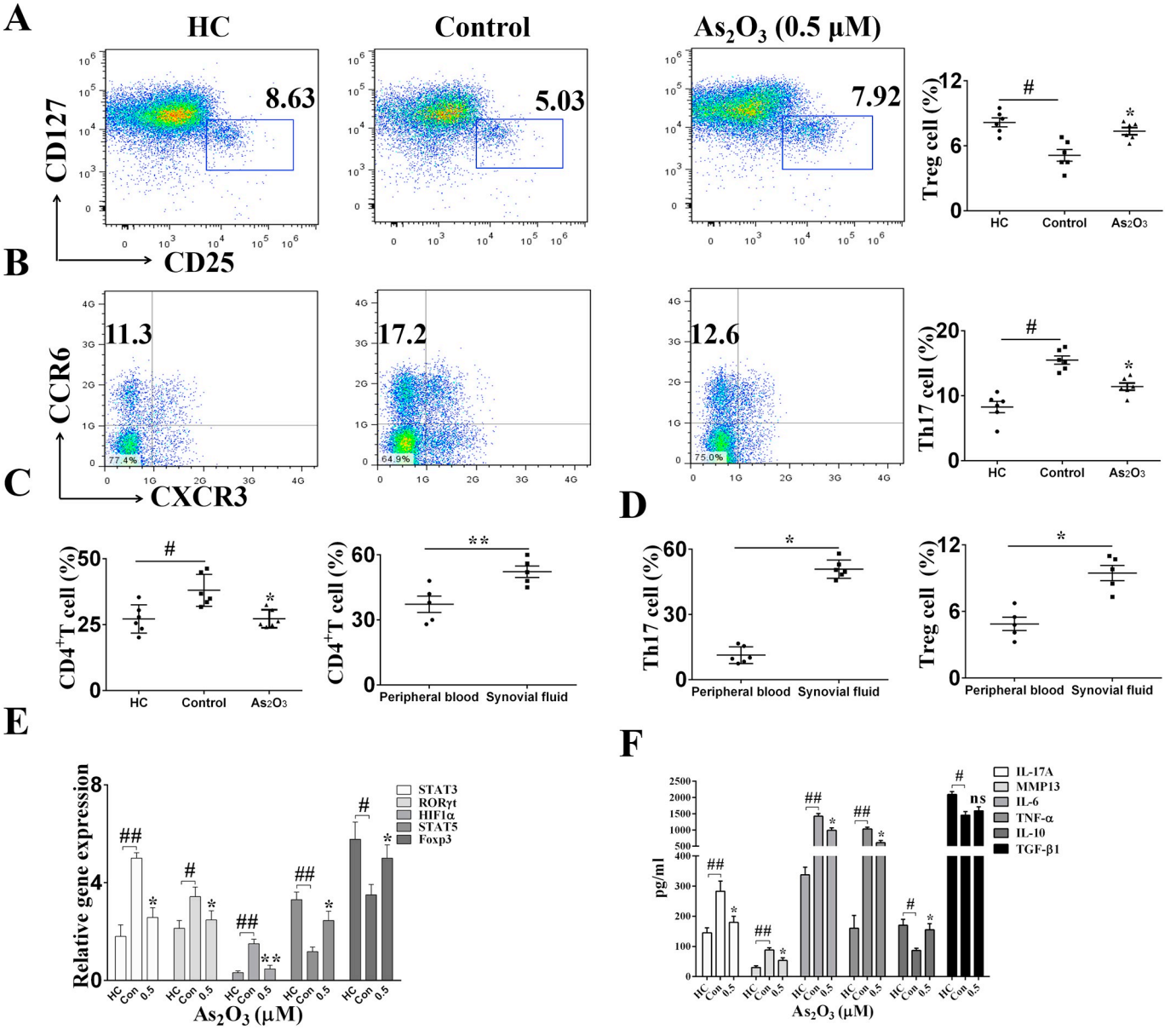

Fig. 2. As_2O_3 inhibited Th17 related signal cytokines while increased Treg cells signal cytokines from activated naïve $CD4^+$T cells. (A and B) Flow cytometric analyses for CXCR3 absence and CCR6 cell surface expression of Th17 cells, CD25 and $CD127^{low}$ cell surface expression of Treg cells obtained from activated $CD4^+$T cells which were isolated from peripheral blood mononuclear cells (PBMCs) of 6 treatment-naïve RA patients and 6 age-and sex-matched healthy controls (HCs). Isotype control staining consistently resulted in 0.1% positive cells throughout the experiments. Numbers are the percentages of cells within the quadrants. (C and D) Quantification of the distribution of the indicated populations within $CD4^+$T cells (C) and the subpopulation of $CD4^+$T cells within the PBMCs (D) of healthy controls and treatment-naïve RA patients. (E) The expression of RORγt, STAT3, HIF1α for Th17 cells, Foxp3, STAT5 and PPARγ for Treg cells were measured by qRT-PCR. (F) The concentration of IL-17, MMP13, IL-6 and TNF-α related with Th17 cells, IL-10 was and TGF-β1 for Treg cells in cell supernatants were determined by ELISA. All of the reactions were performed in triplicate. # $p < 0.05$, # # $p < 0.01$ vs HCs; * $p < 0.05$; ** $p < 0.01$ vs untreated cells (one-way ANOVA).

Th17 lymphocytes express CCR6 in the absence of CXCR3. The population of $CXCR3^-CCR6^+$ expressing Th17 cells was increased in treatment-naïve patients with RA compared with healthy controls (HCs). We next isolated highly purified $CD4^+$T cells separated from PBMCs in this group of patients and HCs. Anti-CD3/anti-CD28 activation of the sorted $CD4^+$T cells population resulted in a relatively large fraction of $CXCR3^-CCR6^+$ expressing Th17 cells (~17.2% upper left) compared with HCs (~11.3%) (Fig. 2B), In contrast, the frequency of $CD25^+CD127^{low}$ expressing Treg cells (~5.03%) were decreased compared with HCs (~8.63%) (Fig. 2A).

Next, we examined whether As_2O_3 could directly affect both Treg and Th17 cell differentiation from naïve $CD4^+$T cells into Th17 and Treg cells. To this end, naïve $CD4^+$T cell were stimulated with anti-CD3/CD28 in the presence of As_2O_3 (0.5 μM). Interestingly, incubation with As_2O_3 (0.5 μM) resulted in a significant reduction in the frequency of Th17 cells (~12.6%) (Fig. 2B). In contrast, the proportion of Treg cells increased to ~7.92% (Fig. 2A).

In addition, a significantly increased $CD4^+$T cell population in the PBMCs of treatment-naïve RA patients compared with HCs was also observed (Fig. 2C). These findings demonstrated that treatment-naïve RA is associated with an increased proportion of $CD4^+$T cells in peripheral blood, mainly caused by the increased frequency of Th17 cells. We further examined Th17 cells and Treg cells in matched peripheral blood and synovial fluid from treatment-naïve RA patients, and observed that the frequency of the two subsets were significantly elevated in synovial fluid (~5.0 fold and 2.0 fold respectively) (Fig. 2D). We also

found that $CD4^+$T cells in synovial fluid were elevated compared with peripheral blood (Fig. 2C). As_2O_3 (0.5 μM) treatment decreased the mRNA expression of RORγt, STAT3 and HIF1α but increased Foxp3, STAT5 and PPARγ mRNA expression compared with anti-CD3/CD28 activated $CD4^+$T cells that had not been treated with As_2O_3 (Fig. 2E). Anti-CD3/CD28 activated $CD4^+$T cells from treatment-naïve RA patients produced significantly more IL-17A in supernatants than did stimulated $CD4^+$T cells from HCs. As_2O_3 had the effect of inhibiting of IL-17A, MMP13, IL-6 and TNF-α to levels similar to those found in HCs (maximal inhibition 95% and 90% respectively for RA patients and HCs) but restored IL-10 (98.2 ± 9.5 pg/mL) to levels similar to those in activated $CD4^+$T cells from HCs (155.3 ± 10.2 pg/mL). Intriguingly, the presence of As_2O_3 had no effect on TGF-β1 production (Fig. 2F).

3.3. As_2O_3 suppresses Th17-producing inflammatory cytokines by transfecting siRNA STAT3

To assess the role of STAT3 in the differentiation of Th17 and Treg cells, we used siRNA knockdown of STAT3 mRNA with special cytokine skewing for Treg and Th17 cells in the presence of As_2O_3 (0.5 μM). Transfection with STAT3 siRNA alone or in combination with As_2O_3 significantly decreased the proportion of IL-17-producing Th17 cells in the anti-CD3/CD28 activated $CD4^+$T cell population isolated from PBMCs in treatment-naïve RA patients (Fig. 3A, B). By contrast, inhibition of STAT3 increased the proportion of cells that were positive for Foxp3, which is the key transcription factor for Treg cell differentiation.

Next, we examined the expression of Th17 transcription factor STAT3 and RORγt genes, and the downstream-related signal cytokines of the Foxp3 and Treg cell pathway, PPARγ, Col2A1 in STAT3-knockdown $CD4^+$T cells. Additionally, STAT3 siRNA suppressed the gene expression of RORγt and STAT3 while STAT3 siRNA increased the gene expression of Foxp3, PPARγ and Col2A1 (Fig. 3C).

We also evaluated the potential impact of As_2O_3 on IL-17, IL-23, MMP13, IL-10 and TGF-β1 production by Th17 and Treg cells. IL-17, IL-23 and MMP13 production were decreased in response to STAT3 inhibition or in the presence of As_2O_3 to the levels seen in activated $CD4^+$T cells from treatment-naïve RA patients, but elevated the IL-10 and TGF-β1 levels produced by Treg cells (Fig. 3D). The changes in the protein level of STAT3 and STAT5 protein in Treg cells from activated $CD4^+$T cells were also evaluated. We found that STAT3 siRNA in the presence of As_2O_3 (0.5 μM) decreased the level of STAT3 but increased the level of STAT5 (Fig. 3E, F).

3.4. As_2O_3 increased Treg cells frequency and reduced Th17 cell frequency by transfecting Lentivirus STAT3

The effect of As_2O_3 on the differentiation of Treg and Th17 cells was explored by using naïve $CD4^+$T-cells isolated from PBMCs of patients with RA. To investigate the mechanism by which As_2O_3 influences naïve $CD4^+$T-cell differentiation, the effect of STAT3 on naïve $CD4^+$T-cell differentiation was investigated. STAT3 expression was enhanced by transfecting lentivirus vector STAT3 (LV-STAT3), and an empty vector (LV-Scramble) as a control in vitro. LV-STAT3 increased the proportion of Th17 cells (~1.2-fold) while it decreased the proportion of Treg cells (~1.5-fold) compared with LV-Scramble. Th17 cells decreased (~1.1-fold) and Treg cells increased (~1.3-fold) after As_2O_3 treatment (Fig. 4A). Additionally, LV-STAT3 upregulated STAT3 and RORγt mRNA expression, but downregulated Foxp3, PPARγ and Col2A1 mRNA expression; however, STAT3 and RORγt reduced after As_2O_3 treatment, whereas Foxp3 and PPARγ and Col2A1 were enhanced in the presence of As_2O_3 (Fig. 4B). As expected, LV-STAT3 decreased the level of IL-10 and TGF-β1 while increasing IL-17A, MMP13 and IL-23 release in culture supernatants. As_2O_3 treatment consistently inhibited IL-17A, MMP13 and IL-23 production while promoting the anti-inflammatory factors IL-10. TGF-β1 production increased slightly and insignificantly (Fig. 4C).

3.5. As_2O_3 ameliorated joint destruction and inflammatory responses in the CIA model

To evaluate the effects of $A_{S2}O_3$ on immuno-inflammation in vivo, a murine CIA model was applied. We compared the effects of $A_{S2}O_3$ with a traditional immuno-inflammation inhibitor, methotrexate (MTX) in CIA mice. After As_2O_3 treatment, the clinical scores and paw swelling decreased (Fig. 5B). The severity of arthritis in the CIA group gradually increased, reaching a plateau on Day 36. $A_{S2}O_3$ (1.0 mg/kg/day) treatment had minimal effects on the severity of joints inflammation. However, joint deterioration in mice treated with $A_{S2}O_3$ at 2.0 mg/kg/day and 5.0 mg/kg/day distinctly slowed from Day 33 (Fig. 5C). Micro-computed tomography with high-resolution scanning also confirmed the disease-modifying effects of As_2O_3. Compared with normal control mice, CIA mice developed massive joint destruction, enlarged joints space and severe bone loss. However, As_2O_3 treatment significantly attenuated CIA-induced joint destruction (Fig. 5D). Assessment of pathologic features revealed that the joints from saline-injected mice with CIA showed proliferation, cartilage damage, pannus formation, and bone erosion (Fig. 5E). In contrast, joints from CIA mice treated with As_2O_3 (2.0 mg/kg/day and 5.0 mg/kg/day) or MTX (2.0 mg/kg/week) showed a remarkable improvement in pathologic findings. Additionally, As_2O_3 significantly decreased the pro-inflammatory cytokines IL-6 and the Th17 cell-related cytokine (IL-17A), whereas Treg cell-related cytokines (IL-10 and TGF-β1) increased, thus indicating that As_2O_3 inhibits inflammatory responses effectively and may also modulate the Treg/Th17 cells balance (Fig. 5F).

3.6. As_2O_3 decreased the proportion of Th17 cells and increased the proportion of Treg cells in the CIA model

The proportion of Th17 cells and Treg cells isolated from spleen tissues were analyzed by flow cytometry to evaluate the Th17-Treg balance in As_2O_3-treated mice. Compared with control mice, As_2O_3 caused a dose-dependent decrease in the percentage of Th17 cells (Fig. 6A). By contrast, As_2O_3 increased the proportion of Treg cells in a dose-dependent manner (Fig. 6B). Not surprisingly, the ratio of Treg/Th17 cells was elevated in the As_2O_3 and MTX-treated groups, especially in the As_2O_3 5.0 mg/kg/day group. Intriguingly, there was no significant difference between the As_2O_3 5.0 mg/kg/day and MTX 2.0 mg/kg/week groups (Fig. 6C). The ratio of Foxp3 to RORγt as well as the expression of Foxp3 mRNA increased, whereas RORγt and STAT3 mRNA expression decreased in the As_2O_3-and MTX-treated groups (Fig. 6D).

3.7. The effects of As_2O_3 on the expression of STAT3, IL-17A, Foxp3 and IL-10 in synovial tissues and spleen tissues

In order to understand the effects of As_2O_3 on the expression of STAT3, IL-17A, Foxp3 and IL-10 in the knee joints and spleen tissues of CIA mice, we performed an immunohistochemical analysis and observed that normal mice showed weak or no immunostaining of STAT3 and IL-17A (Fig. 7A, B, E, F I), but widespread immunostaining of Foxp3 and IL-10 (Fig. 7C, D, G, H I). However, CIA mice receiving saline treatment had significantly increased staining intensity for STAT3 and IL-17A in knee joint synovium and spleen tissues (Fig. 7A, B, E, F II) compared with normal mice, although the staining intensity of Foxp3 and IL-10 decreased significantly (Fig. 7C, D, G, H II) compared with normal mice. Additionally, we found clearly reduced staining intensity for STAT3 and IL-17A in mice receiving the As_2O_3 treatment compared with CIA mice receiving the saline treatment. Immunostaining for STAT3 and IL-17A reduced under As_2O_3 treatment with doses of 2.0 and 5.0 mg/kg/day, almost similar to the effects of MTX (2.0 mg/kg/week) (Fig. 7A, B, E, F III–IV). By contrast, immunostaining intensity for

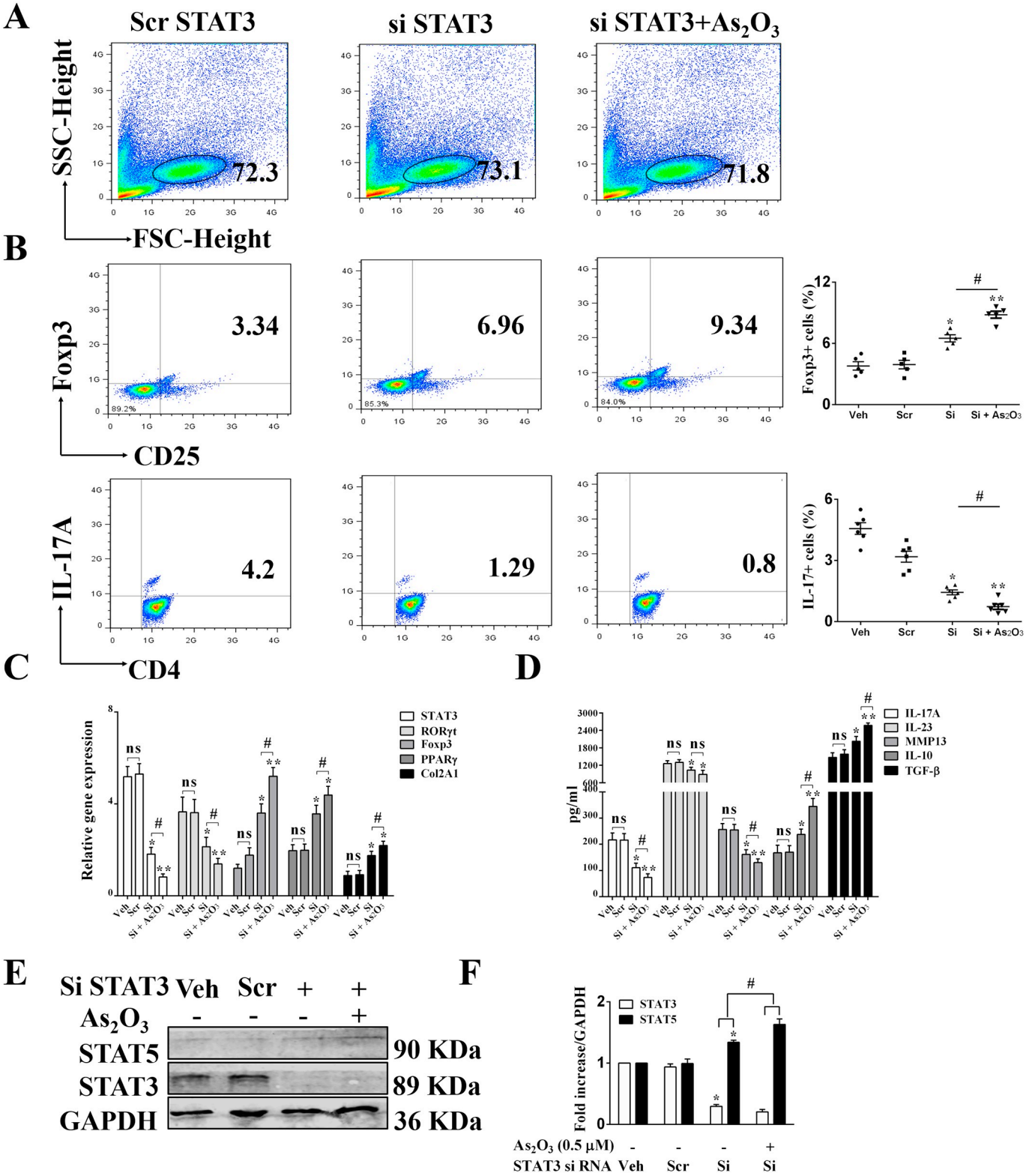

Fig. 3. As_2O_3 affects Th17 and Treg cell differentiation by STAT3-knockdown in naïve $CD4^+$T cells. (A) PBMCs from treatment-naïve RA patients. (B) Dot plots show intracellular staining for Foxp3 and IL-17. Plots are gated on $CD4^+$T cells for Th17 cells and on $CD25^+Foxp3^+$T cells for Treg. The percentage of $Foxp3^+$ Treg cells and IL-17^+ Th17 cells were examined by flow cytometry in anti-CD3/CD28 activated $CD4^+$T cells after transfecting with scramble or STAT3 siRNA or STAT3 siRNA together with As_2O_3 (0.5 μM). (C) qRT-PCR of STAT3, RORγt, Foxp3, PPARγ and Col2A1 mRNA in activated $CD4^+$T cells treated with STAT3 siRNA or control scramble. Knockdown of STAT3 abolished STAT3 mRNA and suppressed the expression RORγt, however, increased the expression of Foxp3, PPARγ and Col2A1 mRNA. (D) Concentrations of IL-17, MMP13 and IL-23, IL-10 and TGF-β1 in culture supernatants of RA peripheral blood mononuclear cells (PBMCs) were measured by ELISA. (E and F) The amount of STAT3 and STAT5 protein in activated $CD4^+$T cells treatment with scramble or STAT3 siRNA or STAT3 siRNA in the presence of As_2O_3 (0.5 μM) examined by western blotting. Densitometry of STAT3 and STAT5 were analyzed by Quantity One software and normalized to GAPDH respectively. The bars represent means ± SEM. # = $p < 0.05$, ## = $p < 0.01$ vs Veh. * $p < 0.05$, ** = $p < 0.01$ vs scramble siRNA. Veh = vehicle control. Scr siRNA = Scramble siRNA.

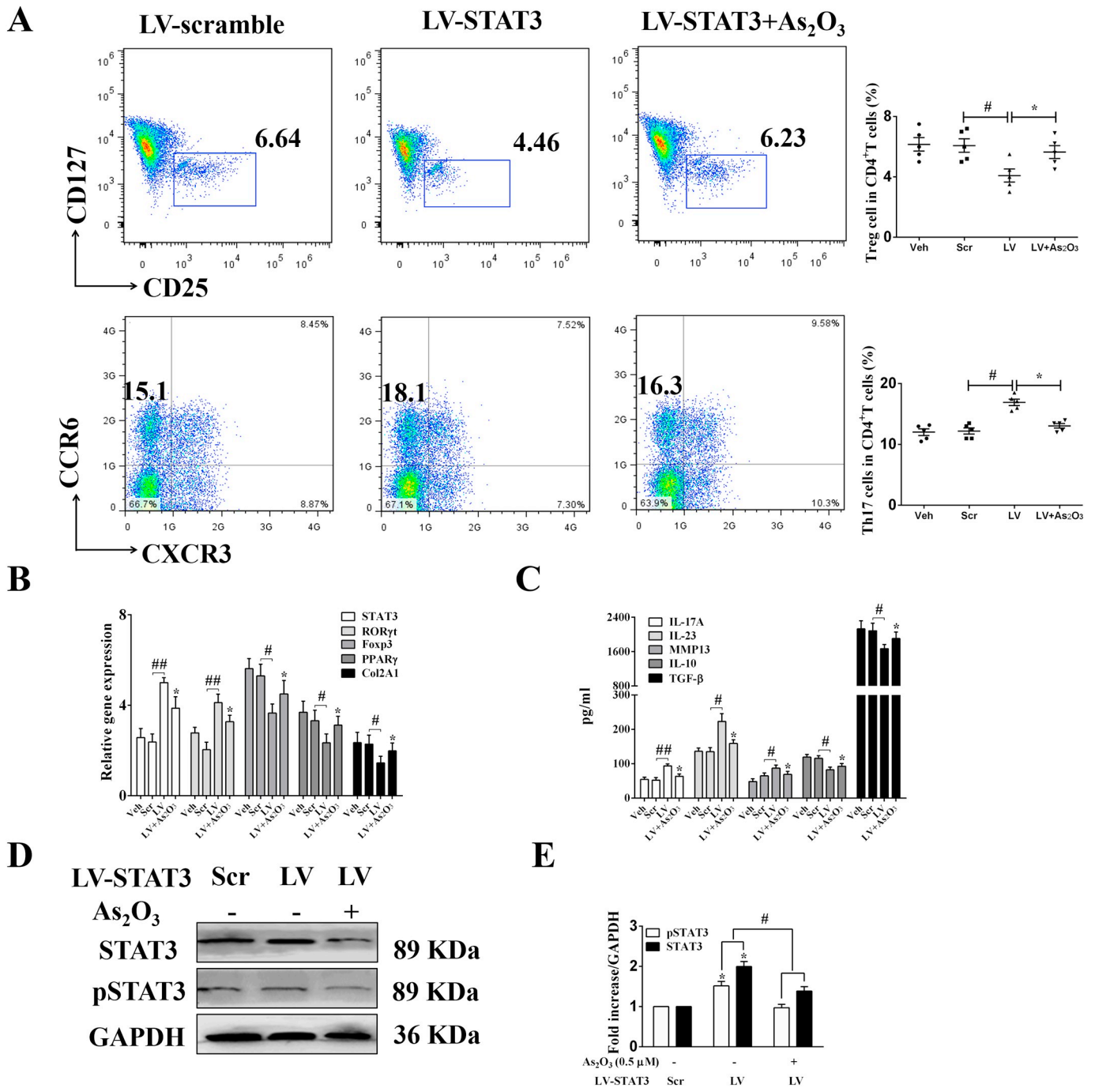

Fig. 4. Effect of As_2O_3 on the differentiation of naïve $CD4^+$T-cells from treatment naïve RA patients by transfecting LV-STAT3. (A) Representative flow cytometric pictures indicated the percentage of Treg and Th17 cells polarized from naïve $CD4^+$T cells transfected with LV-STAT3. (B) The expression of STAT3, RORγt, Foxp3 and PPARγ were determined by qRT-PCR. (C) The concentration of IL-17, IL-23, MMP13 for Th17 cells produced and Th17 related inflammatory cytokines in supernatants, IL-10 and TGF-β1 in supernatants for Treg cells produced were detected by ELISA. All the reactions were conducted in triplicate. (D and E) The protein levels of STAT3 and phosphorylated STAT3 were measured by western blot. Data were represented with mean ± SEM. # $p < 0.05$, ## $p < 0.01$ vs scramble LV-STAT3, * $p < 0.05$, ** $p < 0.01$ vs absence of As_2O_3 group. Veh = vehicle control. Scr Lentivirus = Scramble Lentivirus.

Foxp3 and IL-10 was enhanced under the As_2O_3 2.0 and 5.0 mg/kg/day treatments, as well as the effects of MTX (2.0 mg/kg/week) (Fig. 7C, D, G, HIII–IV).

4. Discussion

The effects of As_2O_3 in acute promyelocytic leukemia and solid tumors have recently been demonstrated. However, the role of As_2O_3 in chronic inflammatory conditions such as RA is still unknown. Our results presented here reveal that As_2O_3, can not only directly modulate the inflammatory responses of already existing and activated Treg and Th17 cells but also critically inhibit their differentiation from activated $CD4^+$T cells by acting on their transcription factor-induced activation mechanisms. Additionally, As_2O_3 can enhance the differentiation of $CD4^+$T cells into Treg cells. Because Treg cells typically serve to dampen excessive immune responses, these cells play an important role in preventing the overreaction of Th17 cells. Although further studies are needed to demonstrate the existence of an indirect modulation of

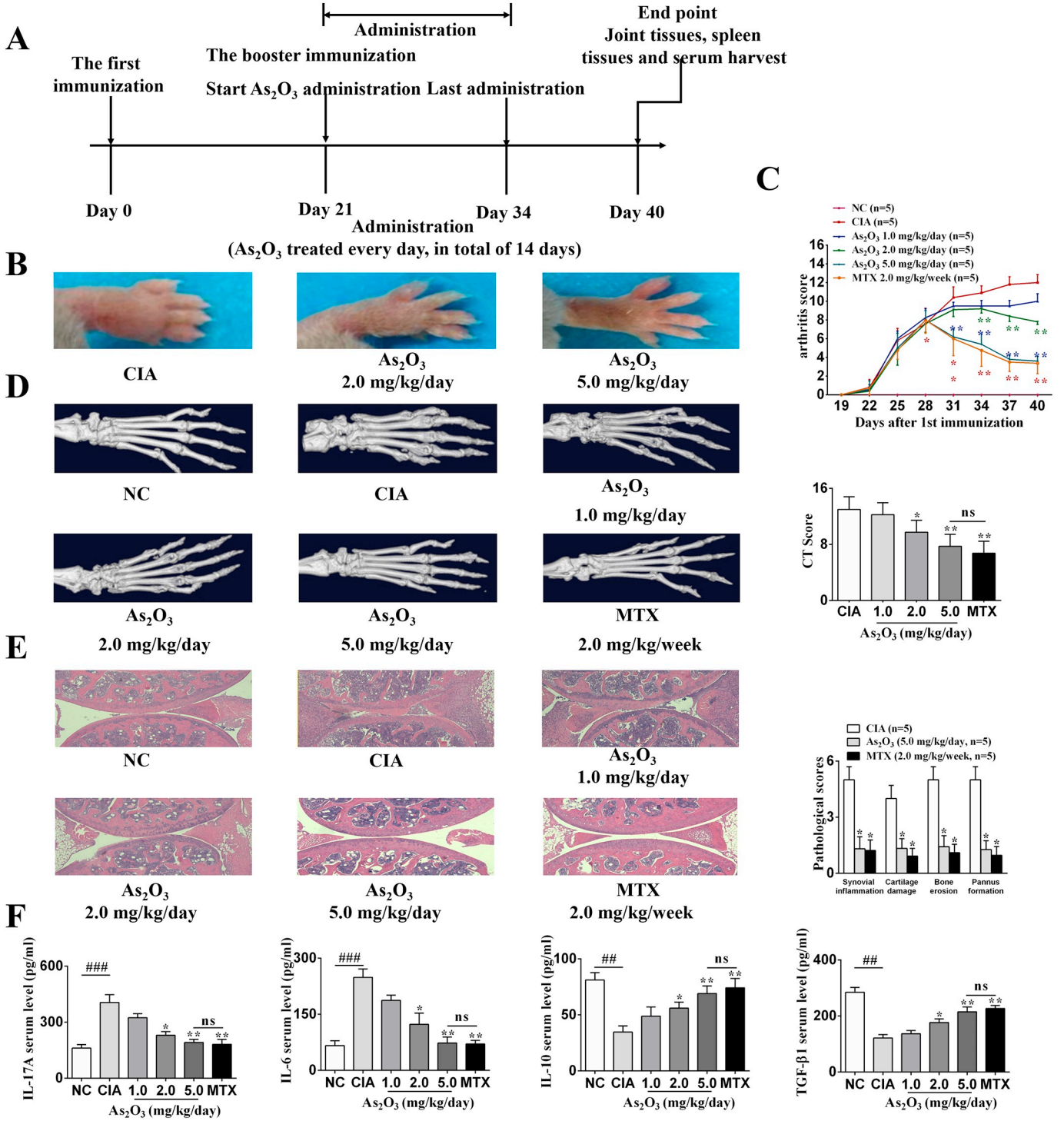

Fig. 5. As_2O_3 prevented the progression of arthritis in the collagen-induced arthritis (CIA) mice. (A) Timeline of As_2O_3 treatment experiment in CIA model. (B) Representative microscopic images of the hind limb from mice were shown. Macroscopic images of mice were taken on day 40 before being sacrificed. (C) Clinical scores of CIA mice during the As_2O_3 treatment. Mice were observed every three days for severity of arthritis by two independent observers who were not aware of the animal's intervention. (D) Mice paws from each group were imaged by a Micro-CT system and representative images were shown. Joint destruction was scored on a scale of 0–4 according to the arthritis severity (n = 5 for each group). (E) Histological score of joints destruction were analyzed. Knee joints of mice were stained by H &E staining. Compared with normal control mice, CIA control mice developed massive joint destruction, enlarged joint space and severe bone loss. However, As_2O_3 treatment significantly attenuated CIA-induced joint destruction. Semiquantitative scores for inflammatory cell infiltration, synovial hyperplasia and bone destruction were assessed by H&E staining, graded on a scale of 0 (normal) to 3 (severe) in 12 scores totally for histological score. (F) IL-17A, IL-6, IL-10 and TGF-β1 were detected by ELISA. All reactions were conducted in triplicate. The bars represent means ± SEM. # $p < 0.05$, ## $p < 0.01$ vs NC, * $p < 0.05$, ** $p < 0.01$ vs CIA control group. NC = normal control group. CIA = CIA control group. MTX = methotrexate. $n = 5$ per group.

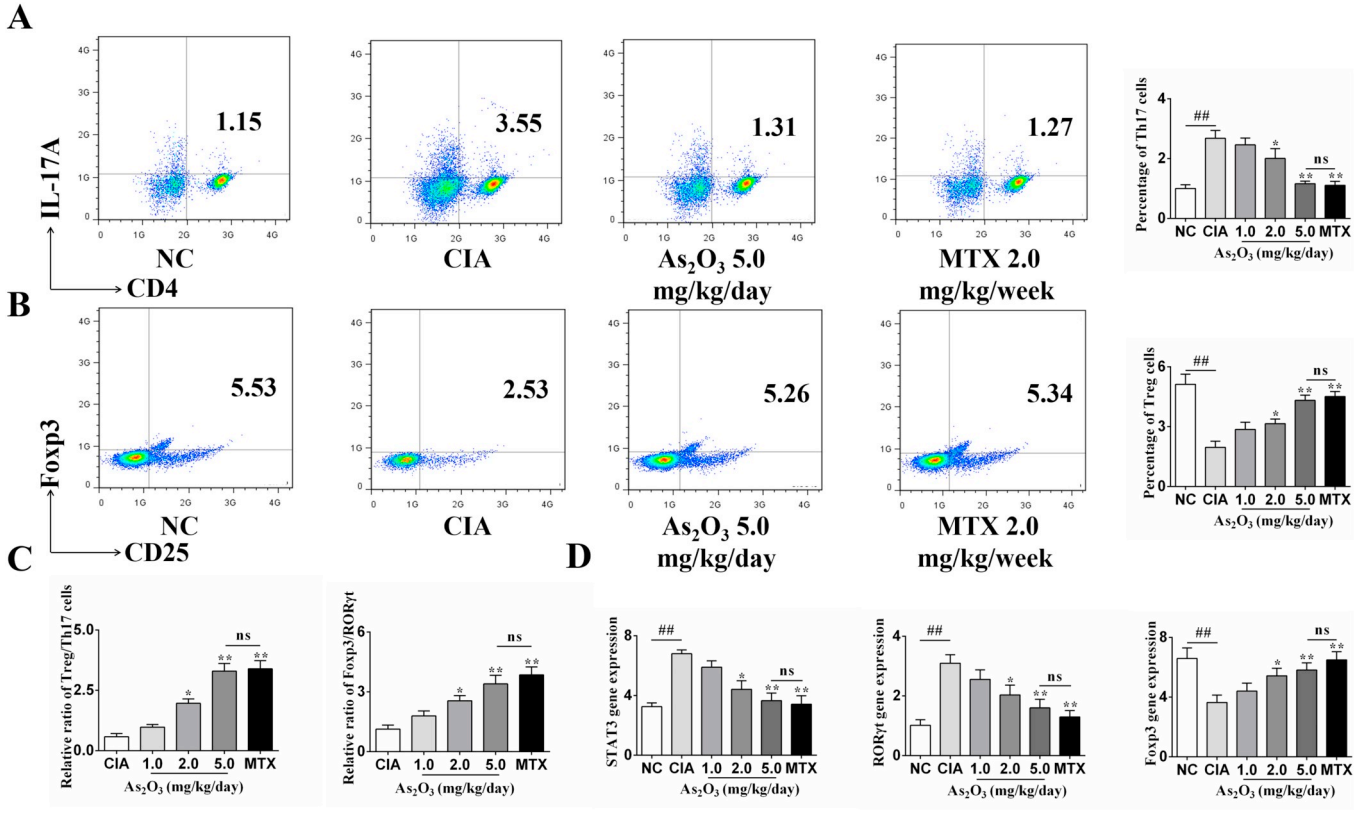

Fig. 6. Effects of As_2O_3 on Th17/Treg cells balance in CIA mice. (A and B) Representative flow cytometric pictures indicated the percentage of Th17 cells and Treg cells in spleen tissues of CIA mice treated with As_2O_3 and MTX. (C) The ratio of Treg/Th17 in spleen tissues of CIA mice treated with As_2O_3. (D) The levels of Foxp3, RORγt and STAT3 mRNA expression in mice spleens from each group were determined by qRT-PCR and normalized to *β-actin*. As well as the ratio of Foxp3/RORγt in primarily cultured splenocytes from spleen tissues of CIA mice treated with As_2O_3 and MTX group was also detected. Data were presented with mean ± SEM. The differences among these groups were assessed by one-way analysis of variance was applied to investigate the differences one by one. n = 5 per group. The bars represent means ± SEM. # $p < 0.05$, ## $p < 0.01$ versus NC, * $p < 0.05$, ** $p < 0.01$ vs CIA control group. NC = Normal control group. CIA = CIA control group. MTX = Methotrexate.

Th17 cells by sustained As_2O_3-mediated induction of Treg cells, these findings suggest that As_2O_3 might modulate inflammatory responses via several selective mechanisms on specific immune cells. This hypothesis is conceivable in light of the recent discovery that Th17 cells trans-differentiate into Treg cells during the resolution of inflammation [30], where As_2O_3 might possibly play a pivotal role in Th17 instability and plasticity.

Zhang and colleagues demonstrated that As_2O_3 can inhibit the Th17-IL-17 axis through inducing $CD4^+$T cells apoptosis in asthma [31]. Haque et al. has demonstrated that As_2O_3 affects the function and survival of Treg cells as well as inducing Treg cells [32]. Moreover, Noack et al. identified the imbalance between Treg and Th17 cells in PBMCs from RA patients, suggesting a relationship between a change in Treg and Th17 cell frequencies and RA [3]. However, the effect of As_2O_3 on Treg-Th17 balance in treatment-naïve RA patients has not been investigated previously. Here, we demonstrated clear differences in the percentage of Treg and Th17 cells and the cytokines related to both subtypes in healthy controls and treatment-naïve RA patients after As_2O_3 treatment. Notably, the data showed that As_2O_3 significantly inhibited Th17 cells differentiation while promoting Treg cells in treatment-naïve RA patients, As_2O_3 also decreased the inflammatory cytokines IL-17A, MMP13, IL-23, IL-6 and TNF-α, while enhancing anti-inflammatory cytokines, such as IL-10 and TGF-β1, suggesting that the imbalance between Treg and Th17 cells is an important factor in the pathogenesis of treatment-naïve RA patients.

Several studies have confirmed the decreasing frequency of Treg cells and the increasing proportion of Th17 cells in treatment-naïve RA patients [33,34]. Our flow cytometry and real-time quantitative polymerase chain reaction data also demonstrated an imbalance of Treg/Th17 in PBMCs from treatment-naïve RA patients. Klotz et al. demonstrated that in the absence of PPARγ, Th17 differentiation increased strongly compared with wild-type $CD4^+$T cells [21]. Vasheghani and colleagues also reported that PPARγ knockdown mice exhibited the accelerated articular cartilage degeneration, which is associated with articular chondrocyte cell death, proteoglycan loss, enhanced expression of catabolic (MMP13) and inflammatory mediators (COX-2 and iNOS) and reduced expression of Extracellular Matrix (ECM) anabolic factors (aggrecan and collagen type II Col2A1) [22].

Recent studies have reported that STAT3 is a key regulator in the process of Treg and Th17 cells exercising their biological function [20,35]. STAT3 has been shown to play a role in regulating the Treg-Th17 cells balance [36,37]. Blockage of STAT3 signaling also inhibited hypoxia-induced HIF1α expression [20]. As expected, after transfection with STAT3 siRNA and LV-STAT3, our results showed that As_2O_3 induced naïve $CD4^+$T to polarize into Treg cells rather than Th17 cells. Furthermore, we demonstrated that As_2O_3 significantly inhibited IL-17A, MMP13, IL-6 and RORγt mRNA in contrast with IL-10, TGF-β1 and Foxp3, which was significantly induced by blocking STAT3. Furthermore, when transfected with lentivirus STAT3, $CD4^+$T cells showed polarization of Th17 cells, suggesting that STAT3 is a downstream gene mediating the restoration of As_2O_3 on Treg-Th17 cell balance in treatment-naïve RA patients (Fig. 8). Nevertheless, the mechanism of the effect of As_2O_3 on Treg-Th17 balance is still unclear. In Th17 cells, STAT3 signaling is essential for differentiation and function. In Treg cells [38], TGF-β1 influences both their differentiation and function, whereas STAT5 regulates their differentiation [39]. Our work has also

shown that the main mechanism by which As_2O_3 exerts its anti-arthritis effect is through regulation of Th17 and Treg cell differentiation and function.

To further examine the potential role of Treg-Th17 imbalance in the pathogenesis of RA, we investigated the effect of As_2O_3 on pro-inflammatory mediators using CIA mice, which closely reflects the in vivo immune environment of RA. As expected, As_2O_3 intervention significantly alleviated the progression of arthritis in CIA mice as reflected

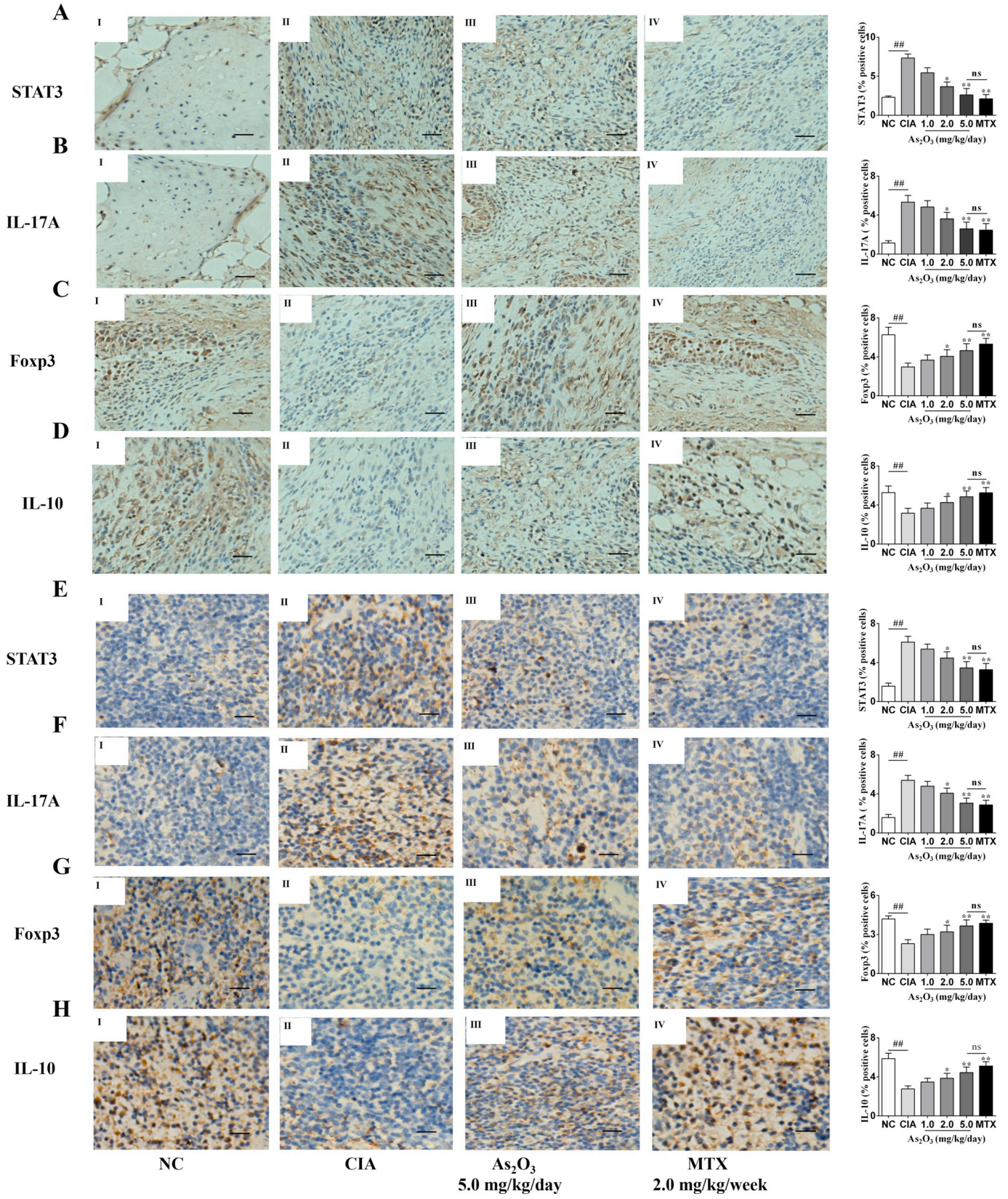

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Fig. 7. As_2O_3 suppressed STAT3 and IL-17A expression and increased Foxp3 and IL-10 expression in synovial tissues and spleen tissues of CIA mice. Positive staining appears as brown color. Immunohistochemical analysis demonstrated increased percentage (%) of positive cells for STAT3 and IL-17A in synovial tissues of CIA mice ($n = 5$; A and B, E and F, II, CIA control mice) compared to synovium of normal mice ($n = 5$; A and B, E and F, I, normal control mice; ## $p < 0.01$), however, positive staining percentage (%) of positive cells for Foxp3 and IL-10 decreased in synovial tissues and spleen tissues of CIA mice ($n = 5$; C and D, G and H, II, CIA control mice) compared to synovium and spleen of normal mice ($n = 5$; C and D, G and H, I, normal control mice; ## $p < 0.01$), while CIA mice treated with As_2O_3 at a doses of 5.0 mg/kg/day ($n = 5$; A and B, E and F, III) and MTX 2.0 mg/kg/week ($n = 5$; A and B, E and F, IV) showed significantly decreased percentage of positive cells for STAT3 and IL-17A in synovial tissues and spleen tissues compared to CIA control mice ($n = 5$; A and B, E and F, II; ** $p < 0.01$). Meanwhile, CIA mice treated with As_2O_3 at a doses of 5.0 mg/kg/day ($n = 5$; C and D, G and H, III) and MTX 2.0 mg/kg/week ($n = 5$; C and D, G and H, IV) showed significantly increased % of positive cells for Foxp3 and IL-10 in synovial tissues and spleen tissues compared to CIA control mice ($n = 5$; C and D, G and H, II; ** $p < 0.01$). Original magnification = ×40. Bars = 25 μM. Data are expressed as the mean ± SEM. # $p < 0.05$, ## $p < 0.01$ versus NC, * $p < 0.05$, ** $p < 0.01$ versus CIA control group. NC = Normal control group. CIA = CIA control group. MTX = methotrexate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

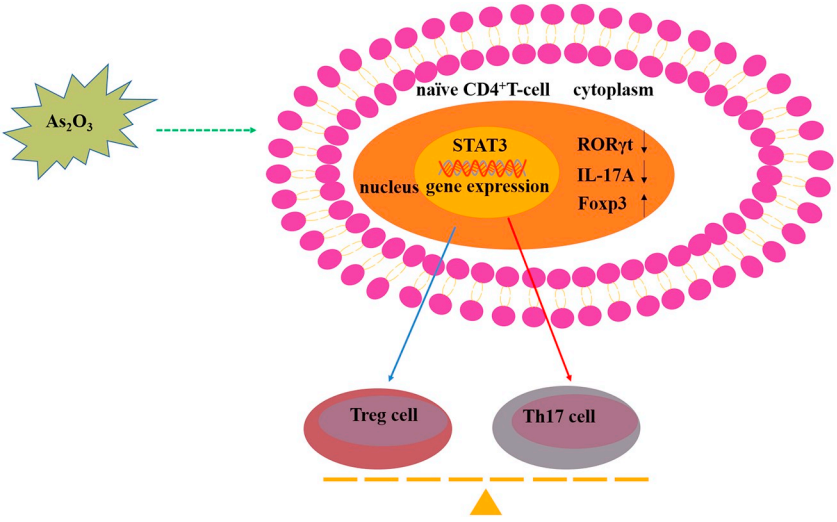

Fig. 8. The mechanism of As_2O_3 regulating Th17 and Treg cells balance through STAT3. As_2O_3 may affect naïve $CD4^+$T cells tended to polarize into Treg cells rather than Th17 cells via modulating STAT3, thus STAT3 is a downstream signaling transcription factor mediating the restoration of As_2O_3 on Treg/Th17 balance in treatment-naïve RA patients.

in the reduction of clinical and histological scores, and remission of the inflammatory response (IL-17A and IL-6 production). Furthermore, a higher proportion of Treg cells and fewer Th17 cells in spleen tissues were detected using flow cytometry after As_2O_3 treatment. Therefore, this study provided a new avenue for the mechanism of action of As_2O_3 in the treatment of RA.

In summary, we have identified a relationship between As_2O_3 and Treg-Th17 balance in treatment-naïve RA patients. We confirmed that As_2O_3 can effectively ameliorate RA progression. The mechanism of alleviation is related to Treg-Th17 imbalance, which is primarily mediated by STAT3. Our data indicate that further insights into the regulation of STAT3 expression and function by As_2O_3 will have profound implications for our understanding of immune function in health and diseases. In CIA mice, active Vitamin D supplementation and Vitamin D analogs can diminish disease activity by reducing IL-17A production [40], Further research into a combination of As_2O_3 and Vitamin D has found that they have a synergistic effect on the inhibition of IL-17A production, as well as attenuating As_2O_3 toxicity (Wang et al, *in press*) [41]. Subsequently, we also investigated the differentially expressed genes in isolated Treg cells from treatment-naïve RA patients in the presence or absence of As_2O_3 through a single cell sequencing technique (unpublished). These data may provide a valuable insight for the development of novel RA precision therapeutic strategies for RA, either as a monotherapy or combination therapy in future.

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Ethics approvals

This study was conducted with the approval of the Harbin Medical University Ethics Committee.

Disclosure statement

The authors have declared that no competing interests exist.

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Contributors

All authors were either involved in the conception and design of the experiment, or analysis and interpretation of data. Each author was involved in drafting the article and revising it critically for important intellectual content. Each author gave their final approval of the version to be published.

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