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## Data Article

## Data on arsenic trioxide modulates Treg/Th17/Th1/Th2 cells in treatment-naïve rheumatoid arthritis patients and collagen-induced arthritis model mice

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## ABSTRACT

In this article, we share the raw protein and mRNA data obtained from basal and stimulated human peripheral blood mononuclear cells (PBMCs) derived from 15 individual treatment-naïve rheumatoid arthritis (RA) patients and synovial fluid mononuclear cells (SFMCs). In treatment-naïve RA patients, PBMCs were treated with a gradient of concentrations of As<sub>2</sub>O<sub>3</sub> (0, 0.1, 0.5, 1.0, 2.0, 4.0 μM) for 48 hours. We found that 2.0 μM As<sub>2</sub>O<sub>3</sub> promoted the apoptosis of PBMCs significantly, and 0.5 μM As<sub>2</sub>O<sub>3</sub> was the lowest and effective concentration that contributed to Treg cell generation but it prevented Th17 cell differentiation, as assessed by flow cytometry. Furthermore, As<sub>2</sub>O<sub>3</sub> decreased the transcription factor STAT3 mRNA expression of Th17 cells but increased the transcription factor Foxp3 of Treg cells. In synovial fluid from RA patients, consistent with PBMCs, As<sub>2</sub>O<sub>3</sub> inhibited Th17 cell differentiation but promoted Treg cell generation. In an animal experiment, we analyzed the body-weight of mice as the indicator of As<sub>2</sub>O<sub>3</sub> toxicity and calculated the spleen index. As<sub>2</sub>O<sub>3</sub> significantly decreased the hematoxylin and eosin score in Type II collagen-induced arthritis in mice. Furthermore, As<sub>2</sub>O<sub>3</sub> downregulated the frequency of Th1 but upregulated Th2 cells. For more insight please see Arsenic

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trioxide improves Treg and Th17 balance by modulating STAT3 in treatment-naïve rheumatoid arthritis patients [1].

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#### Specifications Table

Subject area	Immunology
More specific subject area	Arthritis and Rheumatology
Type of data	Image, graph, figures, histogram.
How data was acquired	Flow cytometry (FC 500, Moflo XDP, Beckman Coulter); Real-time polymerase chain reaction (PCR) (Applied Biochemistry PCR system); Hematoxylin and eosin; immunofluorescence
Data format	Raw and Analysis
Experimental factors	Peripheral blood mononuclear cells (PBMCs) from treatment-naïve rheumatoid arthritis (RA) patients and synovial fluid mononuclear cells (SFMCs) from RA patients were freshly isolated and cultured in standard conditions in the presence of arsenic trioxide ( $As_2O_3$ ); PBMCs and SFMCs were stained with fluorescent antibodies; RNA was extracted from PBMCs; spleen tissues were isolated from DBA/1J mice
Experimental features	The percentage of Treg, Th17, Th1 and Th2 in $CD4^+$ T cells in the presence of $As_2O_3$ was detected by flow cytometry; Treg and Th17 cell-related transcription factors were detected by quantitative real-time PCR.
Data source location	Department of Rheumatology, The First Affiliated Hospital, Harbin Medical University, Harbin, China
Data accessibility	Data are provided with this article
Related research article	Li, C, Zhang, J, Wang, W, Wang, H, Zhang, Y, Zhang, Z, Arsenic trioxide improves Treg and Th17 balance by modulating STAT3 in treatment-naïve rheumatoid arthritis patients, <i>Int Immunopharmacol</i> , 2019,73:539–551. DOI: <a href="https://doi.org/10.1016/j.intimp.2019.05.001">https://doi.org/10.1016/j.intimp.2019.05.001</a>

#### Value of the Data

- The differentiation and function of Treg/Th17/Th1/Th2 cells were affected in the presence of  $As_2O_3$  and this could therefore be an important factor to be considered during research into autoimmune disease. The differentiation and function of Treg/Th17/Th1/Th2 cells were affected in the presence of  $As_2O_3$  and this could therefore be an important factor to be considered during research into autoimmune disease. The differentiation and function of Treg/Th17/Th1/Th2 cells were affected in the presence of  $As_2O_3$  and this could therefore be an important factor to be considered during research into autoimmune disease.
- These data can be compared with other research data emphasizing the role of Treg/Th17/Th1/Th2 cells in the pathogenesis of other diseases.
- The flow cytometry data provides a valuable resource for other researchers to compare the expression levels of a number of Treg/Th17/Th1/Th2 surface markers within the defined  $CD4^+$ T cell populations defined here.
- The data may motivate cancer researchers to investigate the role of Treg/Th17/Th1/Th2 cells in leukemia patients.
- The data of how the  $As_2O_3$  modulates Treg/Th17/Th1/Th2 cells differentiation and function in treatment-naïve RA patients give insight into the immunotherapy and further combination therapy with vitamin D to attain the reduction of side-effects of  $As_2O_3$ .

## 1. Data

All treatment naïve-RA patients related clinical indicators were provided in [Table 1](#) and flow cytometry panels can be referred in [Table 2](#).  $As_2O_3$  significantly promotes the apoptosis of  $CD4^+$ T cells at a concentration of 2.0  $\mu$ M in a dose-dependent manner ([Fig. 1A, B](#), [Table 3](#)). We also detected the effect of  $As_2O_3$  on rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) viability. The data

**Table 1**

Clinical characteristics of treatment-naïve RA patients who provided synovial fluid. PB, peripheral blood; SF, synovial fluid; RF, rheumatoid factor; CCP, citrullinated peptide; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; DAS28, disease activity score 28; TJC, tender joint count; SJC, swollen joint count; VAS, visual analog score.

Patient identifier	Sample	Diagnosis	Gender	Age (yrs)	Age at onset of RA (yrs)	Dis. RF (yrs)	Dur	CCP	ESR	CRP	DAS28 ESR	TJC (28)	SJC (28)	VAS score	Figures
1	PB	RA	F	57	27	30	pos	neg	pos	pos	6.67	11	11	7	1A,B
2	PB	RA	M	48	41	7	pos	pos	pos	pos	6.68	22	0	5	1A,B, 2A–D
3	PB	RA	F	55	54	1	pos	pos	pos	pos	8.19	24	24	7	1A,B
4	PB	RA	F	69	66	3	pos	pos	pos	pos	4.42	0	2	5	2A–D
5	PB	RA	M	41	41	0.5	pos	pos	pos	neg	3.91	2	2	5	2A–D
6	PB	RA	F	55	41	14	neg	pos	pos	pos	7.48	21	21	6	2A–D
7	PB	RA	F	46	39	7	neg	pos	neg	neg	2.16	0	0	5	1A,B
8	PB	RA	F	38	38	0.33	pos	pos	pos	pos	8.21	26	26	7	2A–D
9	PB	RA	M	63	47	16	pos	pos	pos	pos	5.87	10	1	6	2A–D
10	SF	RA	F	67	66	1	pos	pos	pos	pos	5.87	4	4	6	3A,B
11	SF	RA	M	52	51	1	pos	pos	pos	pos	6.66	11	10	7	3A,B
12	SF	RA	F	41	32	9	pos	pos	neg	pos	5.4	6	6	8	3A,B
13	SF	RA	F	48	48	0.58	pos	pos	neg	pos	4.97	2	2	6	3A,B
14	SF	RA	F	61	31	30	pos	neg	pos	pos	5.19	8	2	6	3A,B
15	SF	RA	F	48	48	0.58	neg	pos	pos	pos	5.78	8	8	6	3A,B

**Table 2**

Antibodies and immunostaining panels used for flow cytometry. Detailed description of the fluorochrome-conjugated antibodies and immunostaining panels used to immunophenotype and flow sort the assessed CD4<sup>+</sup>T cell populations.

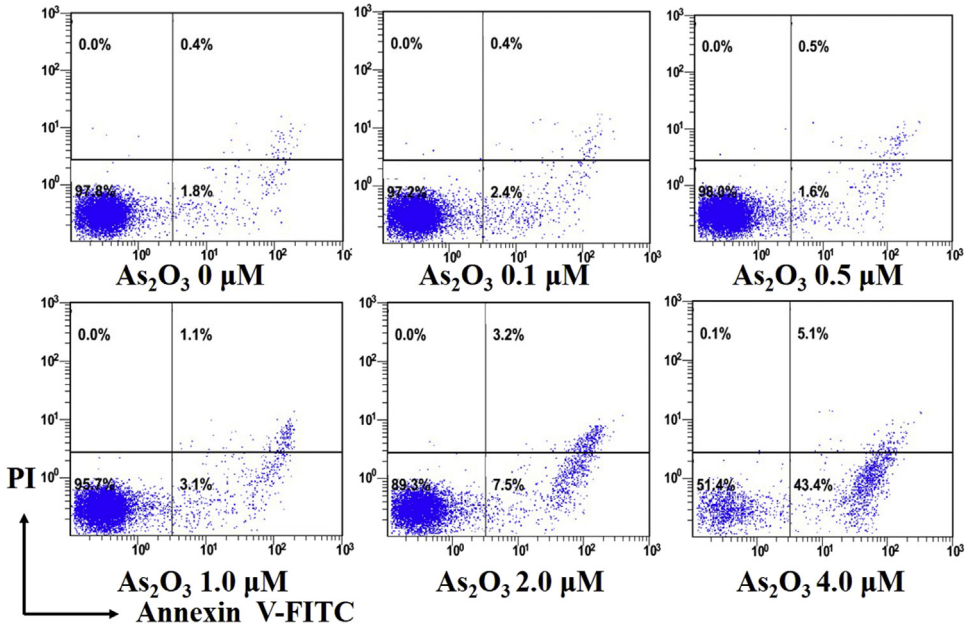
Immunostaining Panel	Antibody	Fluorochrome	Clone	Manufacture
Human Treg Immunophenotyping (Freshly isolated PBMCs)	CD4	FTTC	13B8.2	Becl.r-n Coulter
	CD25	PE	BL49.9	Becl.r-n Coulter
	CD127	PE-Cy5	R34.34	Becl.r-n Coulter
	Foxp3	BV421	206D	Biolezeai
Human Th17 Immunophenotyping (Freshly isolated PBMCs)	CD4	FITC	13B8.2	3eckir.in Coulter
	IL-17	PE	SCPL1362	3eckman Coulter
	CXCR3	PE	G025H7	3iolegend
	CCR6	APC	G034E3	3iolegend
Mouse Th1 Immunophenotyping (Freshly isolated PBMCs)	CD4	FTTC	RM4-5	Biolegend
	IFN- $\gamma$	PE	XMG1.2	Biolegend
	IC	PE	RTK2071	Biolegend
Mouse Th2 Immunophenotyping (Freshly isolated PBMCs)	CD4	FTTC	RM4-5	3iolegend
	IL-4	PE-Cy7	11B11	3iolegend
	IC	PE-Cy7	RTK2071	3iolegend

IC: Isotype control

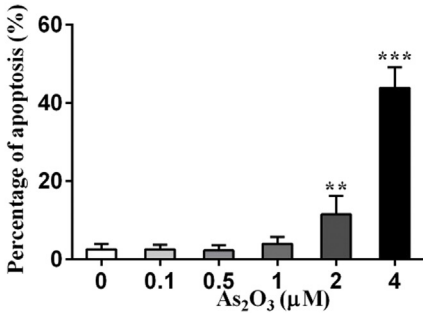
showed that there is no significant change in cell viability (Fig. 1C) [2]. As<sub>2</sub>O<sub>3</sub> at the lowest effective concentration of 0.5  $\mu$ M selectively inhibited the differentiation of CD4<sup>+</sup>T cells into IL-17<sup>+</sup>CD4<sup>+</sup>T cells (Fig. 2A, Table 4) but contributed to Foxp3<sup>+</sup>CD4<sup>+</sup>T cell generation (Fig. 2B, Table 4) in the presence of special polarizing cytokines. Furthermore, in parallel with the previous results, As<sub>2</sub>O<sub>3</sub> downregulated the STAT3 mRNA expression of Th17 cells (Fig. 2C, Table 4) but upregulated the Foxp3 mRNA expression of Treg cells (Fig. 2D, Table 4). Additionally, we also detected Treg and Th17 cells in synovial fluid from RA patients. The percentage of Treg (11.6%) and Th17 cells (55.1%) was higher in synovial fluid (SF) than that in peripheral blood (PB). We found that As<sub>2</sub>O<sub>3</sub> at the concentration of 0.5  $\mu$ M increased the frequency of Treg cells (Fig. 3A) but decreased Th17 cells (Fig. 3B, Table 5).

To further study the effects of As<sub>2</sub>O<sub>3</sub> on CD4<sup>+</sup>T cell proliferation and differentiation, a Type II collagen-induced arthritis (CIA) mouse model was constructed and the groups were divided as described previously [3]. First, we assessed the severity of arthritis from Day 19 after primary immunization according to a standard score system [1]. Second, we used body-weight as the indicator of As<sub>2</sub>O<sub>3</sub> toxicity. As<sub>2</sub>O<sub>3</sub> (1.0, 2.0 or 5.0 mg/kg/day) did not markedly affect the body-weight of CIA mice

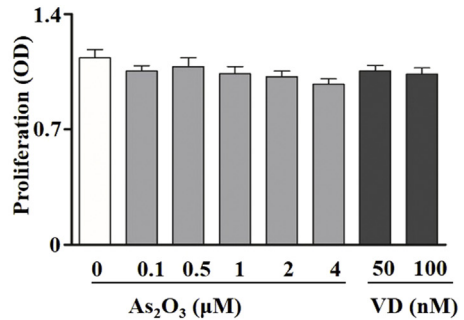
A



B



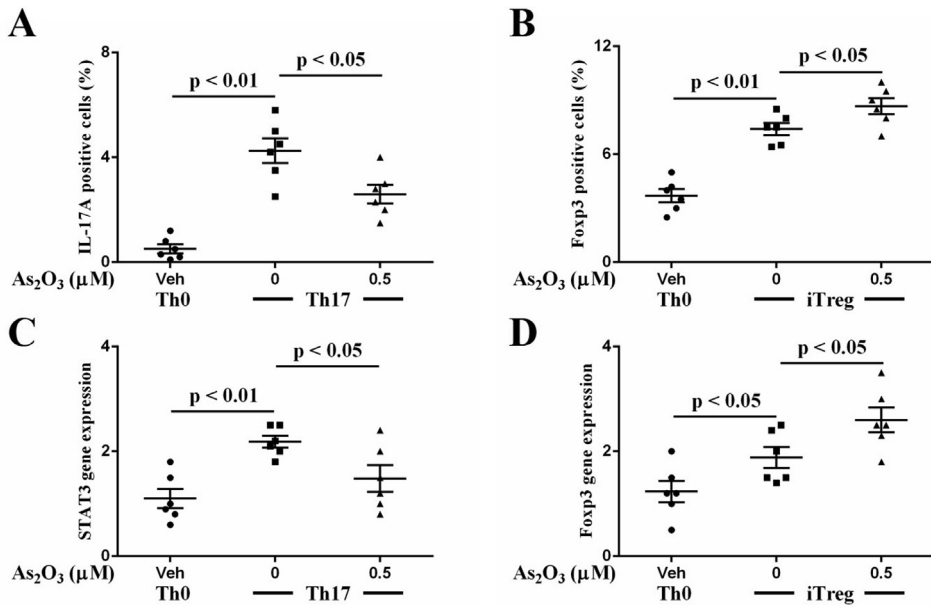
C



**Fig. 1.** As<sub>2</sub>O<sub>3</sub> shows an apoptotic effect on CD4<sup>+</sup>T cells from 2.0 μM. (A). Apoptosis of CD4<sup>+</sup> T cells from treatment-naïve RA patients was detected by flow cytometry. (B). Quantification of the apoptosis percentage. (C). The effect of As<sub>2</sub>O<sub>3</sub> and vitamin D on RA FLS viability, as evaluated by cell count kit 8 (CCK8) assay. \*\*p<0.05 vs non-treatment group, \*\*\*p<0.001 vs non-treatment group by unpaired Student *t*-test. Additionally, mean values, S.E.M and Tukey's multiple comparisons statistics (post hoc) are shown in tabular from below their respective graphs.

**Table 3**The effects of As<sub>2</sub>O<sub>3</sub> on apoptosis of CD4<sup>+</sup>T cells.

Patient identifier	Sample	As <sub>2</sub> O <sub>3</sub> (μM)					
		0	0.1	0.5	1.0	2.0	4.0
		Apoptosis(%)					
1	PB	2.4	1.8	1.6	3.1	7.5	43.4
2	PB	1.6	1.2	1.4	3.8	7.3	48.0
3	PB	3.2	23	2.2	2.5	8.8	41.5
4	PB	2.5	3.0	3.5	5.1	103	50.5
5	PB	5.5	4.5	4.2	7.2	12.1	45.3
6	PB	1.0	2.0	1.2	2.3	15.4	35.7



**Fig. 2.** As<sub>2</sub>O<sub>3</sub> prevented the differentiation of CD4<sup>+</sup>T cells into Th17 cells (A) but promoted Treg cell generation (B). Furthermore, As<sub>2</sub>O<sub>3</sub> downregulated the STAT3 mRNA expression of Th17 cells (C) but upregulated the Foxp3 mRNA expression of Treg cells (D). GAPDH was used as an internal control for normalization real time analysis. The results shown are representative of six independent experiments (n = 6), and the values are expressed as mean ± SEM.

compared with CIA control mice (Table 6). Interestingly, As<sub>2</sub>O<sub>3</sub> reduced the size of spleen tissues and also decreased the spleen index (spleen weight/mice weight) (Fig. 4A, Table 7). Hematoxylin and eosin (H&E) staining revealed that As<sub>2</sub>O<sub>3</sub> (2.0 or 5.0 mg/kg/day) significantly decreased splenic corpuscle proliferation and inflammatory infiltration (Fig. 4B, Table 7). We also found that As<sub>2</sub>O<sub>3</sub> upregulated the percentage of Th2 cells but downregulated Th1 cells in the spleen (Fig. 5A, B, Table 7).

## 2. Experimental design, materials and methods

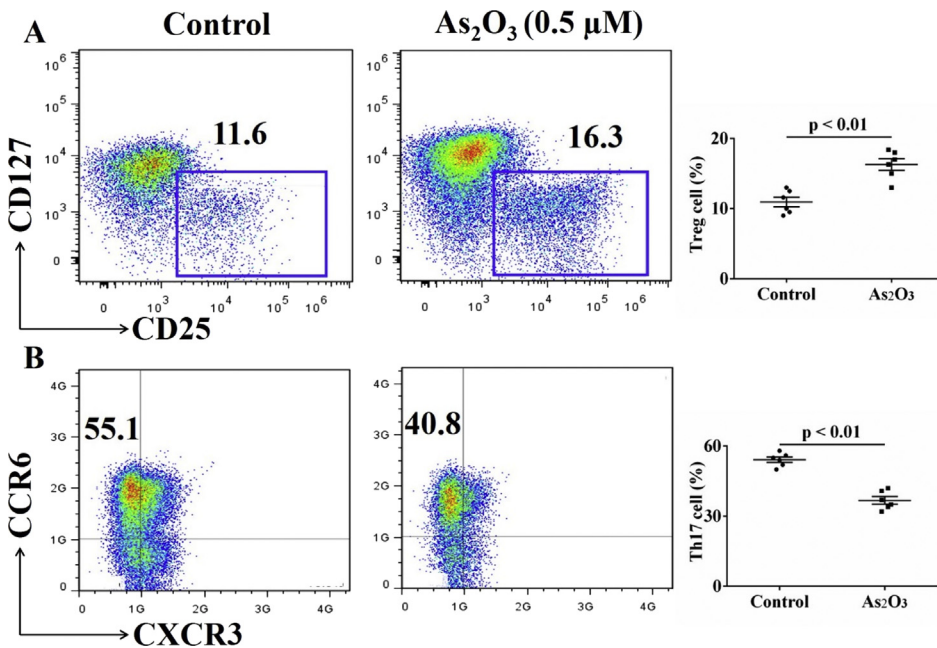
### 2.1. Treatment-naïve RA patients

Treatment-naïve RA patients who fulfilled the American College of Rheumatology 1987 revised criteria [4] were enrolled in the study. Treatment-naïve RA patients were recruited from the

**Table 4**

The effects of As<sub>2</sub>O<sub>3</sub> on the differentiation of CD4<sup>+</sup>T cells under special polarizing cytokines. Th17 cells: Anti-CD3 (2 μg/mL), Anti-CD28 (4 μg/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-23 (100 ng/mL), TGF-β (1 ng/mL). iTreg cells: Anti-CD3 (2 μg/mL), Anti-CD28 (4 μg/mL), IL-2 (20 U/mL), TGF-β1 (2 ng/mL).

As <sub>2</sub> O <sub>3</sub> (μM)	Th17 cells (%)	STAT3 gene expression	Treg cells (%)	Foxp3 gene expression
veh	0.2	0.9	4.2	1.2
	0.5	1.5	2.5	1.5
	0.8	1.0	3.5	1.0
	1.2	0.6	5.0	2.0
	0.1	0.8	3.0	1.2
0	0.3	1.8	4.0	0.5
	4.5	2.2	7.5	1.5
	4.3	2.5	6.4	2.0
	3.5	2.1	8.5	2.4
	5.8	2.5	6.5	2.5
	13	2.0	7.5	1.5
	5.0	1.8	8.0	1.4
0.5	23	2.0	9.0	1.8
	4.0	2.4	9.8	2.3
	2.8	1.5	7.0	2.5
	3.0	1.0	8.0	3.5
	1.5	12	8.5	3.0
	2.0	0.8	9.5	2.5



**Fig. 3.** As<sub>2</sub>O<sub>3</sub> increased the frequency of Treg cells (A) but decreased Th17 cells (B) in synovial fluid from RA patients, as assessed by flow cytometry. The results shown are representative of six independent experiments (n = 6).

Department of Rheumatology of the First Affiliated Hospital of Harbin Medical University. Some patients were taking nonsteroidal anti-inflammatory drugs; none of the patients had been receiving disease-modifying antirheumatic drugs or corticosteroids. The study was conducted with formal approval from the Ethical Committee of Harbin Medical University.

**Table 5**  
The effects of As<sub>2</sub>O<sub>3</sub> on the Treg and Th17 cells from synovial fluid.

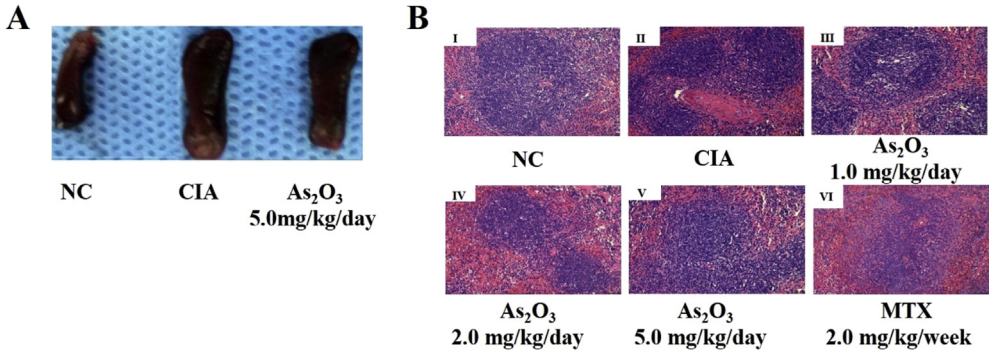
As <sub>2</sub> O <sub>3</sub> (μM)	Percentage (%) (n = 6)	
	Treg	Th17
0	11.6	55.1
	12.5	58.2
	13.4	52.8
	10.5	56.5
	9.5	50.3
	9.4	54.6
0.5	16.3	40.8
	18.5	35.3
	15.4	37.5
	13.3	34.8
	17.5	42.7
	18.4	32.9

**Table 6**  
The effects of As<sub>2</sub>O<sub>3</sub> on body-weight. The body-weight was detected from Day 34 to Day 40. MTX: methotrexate.

Body weight (g) As <sub>2</sub> O <sub>3</sub> (mg kg day) (n = 5)	Days from first immunization						
	34	35	36	37	38	39	40
NC	20.5	20.6	20.7	21.0	21.3	21.7	21.7
NC	19.6	19.7	19.8	19.9	20.1	20.3	20.6
NC	20.3	20.3	21.3	21.3	21.3	21.7	21.7
NC	16.4	16.7	16.9	17.2	17.8	17.9	17.9
NC	17.9	18.0	18.2	18.4	18.5	18.7	18.8
CIA	15.3	15.9	15.7	15.7	16.1	17.0	16.9
CIA	16.3	16.3	15.4	15.5	15.6	16.0	16.4
CIA	16.0	15.9	15.7	15.5	15.6	15.8	16.8
CIA	19.0	18.8	18.2	18.0	18.2	18.1	18.0
CIA	16.8	16.5	16.3	16.3	16.3	16.9	17.3
1.0	20.0	20.1	19.5	19.9	20.2	20.6	21.2
1.0	20.9	20.6	20.4	20.5	21.1	21.4	21.7
1.0	20.8	21.2	20.7	21.2	21.4	21.7	21.8
1.0	17.8	17.7	17.3	18.0	18.5	19.2	19.2
1.0	19.5	19.4	19.4	19.2	19.2	19.0	19.1
2.0	16.6	16.1	16.4	16.2	17.4	17.4	18.1
2.0	20.6	20.7	19.9	20.1	20.3	20.7	20.5
2.0	19.0	18.7	19.3	18.5	18.7	19.1	19.2
2.0	18.8	18.2	18.0	18.3	18.8	19.5	19.2
2.0	19.5	19.2	18.9	19.0	20.2	20.0	20.1
5.0	16.9	17.3	16.7	17.6	17.4	18.0	18.5
5.0	20.6	17.7	17.6	16.9	17.6	17.7	17.5
5.0	18.7	20.8	20.8	21.2	20.8	21.2	21.5
5.0	16.8	17.3	17.2	17.8	18.0	18.2	19.3
5.0	19.2	20.7	20.7	21.5	21.4	22.0	21.9
MTX	16.9	17.1	17.4	18.1	18.5	19.4	19.6
MTX	17.9	20.5	21.4	21.9	22.0	22.0	21.7
MTX	21.	18.8	19.1	19.0	19.6	20.1	20.2
MTX	17.6	16.8	16.6	17.1	17.2	17.9	17.9
MTX	20.5	19.1	19.3	19.5	19.4	19.2	19.3

## 2.2. Peripheral blood and synovial fluid mononuclear cell isolation of naïve CD4<sup>+</sup>T cells

PBMCs were isolated from treatment-naïve RA patients and healthy volunteers. Synovial fluid mononuclear cells were isolated from synovial fluid from RA patients, then separated by density



**Fig. 4.** The effects of  $As_2O_3$  on spleen tissues. (A).  $As_2O_3$  decreased the volume of the spleen. (B). The spleen index in the CIA control group increased significantly compared with the normal control group. However, the spleen index of groups under  $As_2O_3$  (2.0 or 5.0 mg/kg/day) and MTX treatment decreased significantly compared with the CIA control group. Splenic corpuscle proliferation and splenic sinus congestion in the CIA group increased significantly compared with the normal control group. However, splenic corpuscle proliferation and splenic sinus congestion in the  $As_2O_3$  and MTX treatment groups decreased significantly compared with the CIA control group.

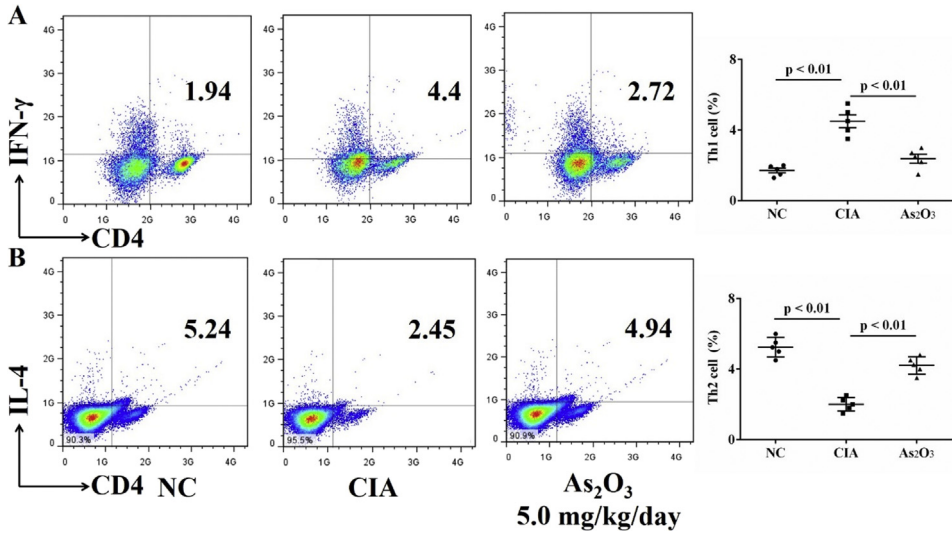
**Table 7**

The effects of  $As_2O_3$  on spleen tissues. Percentage of Th1 and Th2 cells in spleen lymphocytes. Were detected by flow cytometry.

Group (n = 5) As.Oj (mg kg day)	Index of spleen	Spleen Histological score	Th1 (%)	Th2 (%)
NC	2.1	0.8	1.9	5.2
NC	2.2	1.1	1.5	6.0
NC	1.9	1.2	2.0	5.5
NC	1.8	1.0	1.8	4.5
NC	2.0	1.3	1.3	5.0
CIA	4.8	2.7	4.5	2.2
CIA	4.6	2.6	5.0	2.0
CIA	5.1	2.5	4.0	1.5
CIA	5.2	3.2	5.5	2.5
CIA	4.5	3.5	3.5	1.8
1.0	4.0	2.3	4.3	2.8
1.0	4.3	2.4	5.0	2.3
1.0	4.5	2.1	3.5	2.0
1.0	4.2	2.8	4.8	3.5
1.0	3.9	3.0	3.0	3.2
2.0	3.6	1.8	3.5	3.8
2.0	3.9	2.0	4.0	2.9
2.0	4.0	1.7	3.0	3.0
2.0	4.2	2.0	4.2	3.5
2.0	3.2	2.8	3.0	4.0
5.0	3.4	1.4	2.7	4.2
5.0	3.2	1.1	2.5	4.5
5.0	3.0	1.7	3.0	4.0
5.0	3.5	1.2	1.5	3.5
5.0	2.9	2.0	2.2	4.8
MTX	2.6	1.2	2.5	4.8
MTX	3.0	1.3	2.2	4.3
MTX	2.8	1.5	3.0	3.9
MTX	3.2	1.8	1.4	5.2
MTX	2.7	1.0	2.1	5.0

gradient on a Ficoll-Hypaque apparatus (MD Pacific Biotechnology, Tianjin, China). Naïve  $CD4^+T$  lymphocytes were purified on a high-speed cell sorter system (Moflo XDP, Beckman Coulter, Indianapolis, IN, USA). The naïve  $CD4^+T$  cells had a purity of >95%, which was confirmed by flow cytometry (FC500, Beckman Coulter, Indianapolis, IN, USA).





**Fig. 5.** As<sub>2</sub>O<sub>3</sub> decreased the frequency of Th1 cells (A) but increased the proportion of Th2 cells (B) in the spleen of DBA/1J mice, as assessed by flow cytometry. The results shown are representative five independent experiments, and the values are expressed as mean ± SEM.

### 2.3. Apoptosis assay

Sorted live CD4<sup>+</sup>T cells from treatment-naïve RA patients were cultured in the presence of different concentrations of As<sub>2</sub>O<sub>3</sub> for 48 hours. Apoptosis was determined by Annexin V and propidium iodide staining according to the manufacturer's protocol (BD Biosciences, San Diego, CA, USA). When gated on total cells, Annexin V- and propidium iodide-negative cells were considered as living. No significant changes in total cell numbers were observed. Samples were analyzed by flow cytometry (FC 500, Beckman Coulter, Indianapolis, IN, USA).

### 2.4. In vitro stimulation polarization of CD4<sup>+</sup>T cells

CD4<sup>+</sup>T cells were cultured with anti-CD3 (2 µg/mL)/anti-CD28 (4 µg/mL) (Biolegend, San Diego, CA). IL-1 β (10 ng/mL), IL-6 (20 ng/mL), TGF-β (1 ng/mL) (Biolegend, San Diego, CA), IL-23 (100 ng/mL) (R&D Systems, Minneapolis, USA) were added for Th17 cell polarization; TGF-β (2 ng/mL) and IL-2 (20 U/mL) (Biolegend, San Diego, CA) were added for Treg cell polarization. At the same time, As<sub>2</sub>O<sub>3</sub> (0.5 µM) (Yitaida Pharmaceutical Factory, Harbin, Heilongjiang, China) was added once a day for 3 days.

### 2.5. In vitro proliferation assay

The cell proliferation assay was evaluated with a Cell Counting Kit-8 (Sigma, St Louis, MO, USA) following the procedures described earlier with minor modifications [5]. Briefly, 10<sup>4</sup> cells were seeded in a 96-well plate. After 24 h, different concentrations of drugs or vehicles were added with fresh medium. Cells were incubated at 37 °C for 48 h before detected at 450 nm immediately. The experiments were repeated three times.

### 2.6. Flow cytometry analysis

For intracellular cytokines detection, cells were stimulated with the corresponding cell activation cocktail (with Brefeldin A) (Biolegend, San Diego, CA) for 6 h. After surface staining for 15 min, the cells

were resuspended in a fixation buffer, and 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. Isotype control staining consistently resulted in 0.1% positive cells throughout the experiments. 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), allophycocyanin-conjugated CCR6 (clone: G034E3) and phycoerythrin-conjugated CXCR3 (clone: G025H7). 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-IFN- $\gamma$  phycoerythrin (clone: XMG1.2) and anti-IL-4-PC7 (clone: 11B11). All these antibodies were purchased from Biolegend (San Diego, CA, USA).

### 2.7. Real-time quantitative polymerase chain reaction analysis

The methods used for RNA extraction and cDNA synthesis have been described previously [3]. Data were normalized to *glyceraldehyde-3-phosphate dehydrogenase* and  $\beta$ -*actin*, and were calculated via the  $2^{-\Delta\Delta Ct}$  method.

### 2.8. Animal model and experimental protocol

Male DBA/1 J mice (5–8 weeks old, 20 g  $\pm$  2 g in weight; Charles River, Beijing, China) were housed under specific pathogen-free 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 [3]. The scoring system was used according to a protocol described previously [6]. The mice were carefully monitored for toxicity symptoms and weighed every 3 days. The mean body-weight and spleen index of each group were calculated and used as a parameter of toxicity.

### 2.9. Histology analysis

Mice spleens were collected after sacrifice. The procedures of fixation and embedding have been described previously [3]. In brief, the spleens were embedded in paraffin, cut into 5- $\mu$ m sections and stained with H&E. Histology scores were assessed as previously described [3].

### 2.10. Spleen lymphocyte preparation

Mice were sacrificed, then spleens were drained and by passing through a 200  $\mu$ m wire mesh to obtain single-cell suspensions. PBMCs were isolated by Ficoll density gradient isolation (Haoyang, Tianjin, China) according to the manufacturer's instructions. Mononuclear cells were obtained and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL of penicillin, 100  $\mu$ g/ml of streptomycin, nonessential amino acids, 1  $\mu$ M sodium pyruvate and 2 mM L-glutamine. Subsequently, flow cytometry detected the percentage of Th1 and Th2 cells.

### 2.11. Statistical analysis

The data were analyzed by using GraphPad Prism Software (Version 6 for Windows; Graphpad Prism, San Diego, CA, USA). Simple comparisons were conducted with unpaired, two-tailed Student's *t*-test for parametric data. Values of  $P < 0.05$  were considered statistically significant. All data were expressed as mean  $\pm$  S.E.M.

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## Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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