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ORIGINAL RESEARCH

Total Alkaloids of *Sophora alopecuroides* Linn. Attenuates Rheumatoid Arthritis Through Regulating Follicular Helper T Cells

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Background: Rheumatoid arthritis (RA) is a chronic autoimmune disease with abnormal differentiation of follicular helper T (Tfh) cells, Total alkaloids of *Sophora alopecuroides* Linn. (Leguminosae) (TASA) have potential effects on collagen-induced arthritis (CIA) mice, while the mechanism needs further elucidation. The purpose of this study is to explore the regulation of TASA on rheumatoid arthritis and related mechanism.

Methods: The proportion of Tfh and B lymphocytes in peripheral blood lymphocytes of RA patients was examined by flow cytometry. We constructed the collagen induced arthritis DBA/1J mice model. Between days 15 and 45 following the first immunization, the mice were treated intraperitoneally with saline, TASA (100, 50, and 25 mg/kg), and dexamethasone (DXM) for 30 days. Molecular biological techniques such as FCM, PCR, ELISA, and Western-blotting were used to examine Tfh cells and associated signal pathways.

Results: Our results indicated that the follicular helper T cells and B lymphocytes in rheumatoid arthritis patients were significantly increased compared with the healthy control. The percentage of Tfh cells are correlated with RA related inflammatory factors. Total alkaloids of *Sophora alopecuroides* Linn. could significantly attenuate joint swelling. Meanwhile, it reduced the frequencies of spleen Tfh, B lymphocytes and the expression of TLR2, TLR9, p-NF-κBp65, CXCR5, Bcl-6, ICOS of ankle joints in CIA mice.

Conclusion: Total alkaloids of *Sophora alopecuroides* Linn. may down-regulate the frequency and function of Tfh cells and inhibit GCB cells via TLRs/NF-κB signal pathway to relieve the immune-pathological progression of CIA mice.

Keywords: collagen-induced arthritis, C-X-C chemokine receptor type 5, total alkaloids of *Sophora alopecuroides* Linn., toll-like receptors

Introduction

Rheumatoid arthritis is an autoimmune disease involving multiple joints, which is mainly characterized by the erosion of bone tissue and joint damage, and even lowers life expectancy.[1] With the development of immunology, $CD4^+$ T cells[2], fibroblasts (such as synovial cells), macrophages, B lymphocytes and their secreted autoantibodies have been found to be participating in the morbidity of rheumatoid arthritis, but it's pathogenic mechanism is unclear.

Follicular helper T (Tfh) cells are a newly discovered subset of $CD4^+$ T cells that help B lymphocytes perform humoral immunity. The phenotype of Tfh is $CD4^+CXCR5^+ICOS^+$, which expresses cytokines IL-21.[3] The central role of Tfh cells is to promote the formation of high-affinity B lymphocytes and plasma cells. It also plays a crucial role in the development of B-cell antibody affinity maturation, high-frequency mutations, class switching, and memory B-cell formation.[4] A large number of reports have shown unconstrained amplification of Tfh cells in CIA models and rheumatoid arthritis patients,[5] indicating that Tfh cells and their secreted cytokines exhibit a critical role in the pathogenesis of rheumatoid arthritis.

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TLRs (toll-like receptors) are types of protein involved in the innate immune response.[6] They are mainly expressed in synovial fibroblasts and macrophages, and play an essential part in activating the immune reaction and serve as a link between the adaptive and innate immune response. A great majority of studies have shown that TLR2 and TLR4 are related to the pathogenesis of rheumatoid arthritis.[7] Compared with normal people, TLR2 and TLR4 in rheumatoid arthritis patients are significantly increased. This indicates that the TLRs/NF-κB signaling pathway plays a significant role in autoimmune diseases, but the exact mechanism of the TLRs/NF-κB signaling pathway in rheumatoid arthritis is not yet clear.

Total alkaloids of *Sophora alopecuroides* Linn. (TASA) are a mixture of extract from the medicinal plant *Sophora alopecuroides* Linn. (Kudouzi), which usually grows in northwest China.[8] In China, The total alkaloids of *Sophora alopecuroides* Linn. mainly contains matrine, sophoridine, oxymatrine, oxysophocarpine, sophocarpine and sophoramine.[9] As an important modern Chinese medicine, TASA has significant effects, especially anti-inflammatory, anti-apoptosis, anticancer and regulation of immunity.[10] However, there are few reports on the study of TASA relieving rheumatoid arthritis. In the current study, our target is explore the response of Total alkaloids of *Sophora alopecuroides* Linn. in CIA mice and the immunomodulatory activities mediated by TLR/NF-κB signal transduction pathway in Tfh cells.

Materials and Methods

Patients

This research included in 30 rheumatoid arthritis patients from the Affiliated Hospital of Ningxia Medical University (the classification standard of the American Rheumatology Society revised in 1987 was used to diagnose rheumatoid arthritis) and 20 healthy controls. EDTA anticoagulation from the residual peripheral blood of the medical laboratory was used in this study. The clinical indicators of patients and healthy controls are shown in Table 1 (Table 1). The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of Ningxia Medical University, Yinchuan, China (2021 - G003).All study subjects signed informed written consent forms.

Sample Processing and Flow Cytometry

EDTA (3 mL) anticoagulant blood was gathered from patients and healthy controls respectively. Separation of White Blood Cells (WBC) with Human Peripheral Blood Erythrocyte Lysate. WBCs at 5×10^6 /tube were marked with CD3-eFluor 450, CD4-eFluor 506, CXCR5-FITC, ICOS-Alexa Fluor 700, CD19-APC for 30 min in 4°C. After cleaning with stain buffer, the cells were analyzed by Beckmann flow cytometry.

Animals

Six-week-old male DBA/1J mice were bought from Beijing Weitong Lihua Co., Ltd.

The experimental mice were housed under a 12 h light/dark cycle and temperature and humidity-controlled environment, and food and water were supplied *ad libitum*.

Table 1 Clinical Characteristics of Healthy Controls and RA Patients

	RA	HC
Age (year)	50.87±15.96	53.2±9.89
RF (IU/mL)	75.89±81.83	7.43±4.01
CCP (>100U/mL)	20/30	–
CRP (mg/L)	26.67±29.33	1.425±1.658
IgA (g/L)	3.27±1.26	2.82±1.17
IgM (g/L)	2.09±3.57	1.18±0.73
IgG (g/L)	15.4±3.81	13.27±2.52
C3 (g/L)	1.18±0.28	–
C4 (g/L)	0.24±0.12	–
IL-21 (pg/mL)	100.6± 22.27	18.18±4.065
IL-23 (pg/mL)	16.61±3.033	18.31±4.094
IL-10 (pg/mL)	267.2±35.89	431.2±67.72

Before starting, All experimental operations were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Laboratory Animal Ethics Committee of Ningxia Medical University (SCXK (Jing) 2019–0008).

Drugs

Total alkaloids of *Sophora alopecuroides* Linn. was bought from Ningxia Zijinghua Pharmaceutical Co., Ltd., The content of aloperine, sophoridine, oxymatrine, oxysophoridine, matrine, sophocarpine in Total alkaloids of *Sophora alopecuroides* Linn. was examined by HPLC.

Induction of CIA Model and Arthritis Assessment

Six-week-old male DBA/1J mice were randomly divided into six groups (n = 6): CIA model group, dexamethasone positive control group, normal control group, high dose TASA group (100 mg/kg/day), medium dose TASA group (50 mg/kg/day) and low dose TASA group (25 mg/kg/day). On the first day, the animals were immunized subcutaneously with bovine type II collagen and complete Freund's adjuvant. On the 21st day, collagen and incomplete Freund's adjuvant were emulsified and injected subcutaneously near the back. Between days 15 and 45 following the first immunization, the mice in the TASA-treated group were administered with Total alkaloids of *Sophora alopecuroides* Linn. at different concentration. The dexamethasone group was given a dose of 2.8 mg/kg from day 15 to day 21, and a dose of 1.4 mg/kg was given after day 21, once a day, with each dose of 0.1mL. The normal control group was injected with 0.1mL of physiological saline every day until the 45th day. Arthritis, foot swelling, weight and joint score of mice were evaluated by the same person from the first day to the 45th day.

Histological Analysis of Arthritis

On day 45 after the first immunization, DBA/1J mice were sacrificed. Calculate spleen index. The joints and spleen were fixed in 4% formaldehyde solution for 24 h, and the joint was decalcified in formalin EDTA decalcifying solution (G2520, Solarbio) for 1 month for fresh solution once a week. Then the joint tissue was embedded and sliced. The joint histopathology was studied by HE staining (Sl107-2, SEVEN).

Enzyme-Linked Immunoassays (ELISAs) of Inflammatory Cytokines

The levels of IL-10 (JL19246,Shanghai Jianglai Industrial Limited By Share Ltd.), IL-21 (BY-EH110354,Nanjing Boyan Biotechnology Co., Ltd.) in human peripheral blood serum, and the expression of IgG1 (CEA074Mu, Cloud-clone Corp. Wuhan), IL-21 (JL20239, Shanghai Jianglai Industrial Limited By Share Ltd.), IgG2a (SES094Mu, Cloud-clone Corp. Wuhan), type II collagen-specific antibodies (JL20402, Shanghai Jianglai Industrial Limited By Share Ltd.) in mouse serum were detected by ELISA according to the manufacturer's instructions.

Flow Cytometry Analysis

Spleen lymphocytes were isolated from CIA mice. Suck the white film layer into a 15mL centrifuge tube (601001,NEST Biotechnology) and add an equal amount of PBS buffer (C3590-0500, VivaCell) for washing. Resuspension spleen lymphocytes with staining buffer (E-CK-A107, Elabscience) and labeled with CD4, CXCR5, ICOS, B220, GL-7. The percentage of spleen $CXCR5^+CD4^+ICOS^+$Tfh cells and $B220^+GL\text{-}7^+$ B lymphocytes was analyzed on the BD Accuri C6.

Sort Spleen $CD4^+$ T Cells and Induction to Tfh Cells

We isolated spleen $CD4^+$ T cells following the instructions of STEMCELL mouse $CD4^+$ T cell sorting kit (Catalog: 19852A) and cultured 5×10^5 $CD4^+$ T cells per well in a 12-well plate. The 12-well plate was pre-coated with 4 μg/well anti-CD3 and 4 μg/well anti-CD28 overnight before sorted. The $CD4^+$ T cells were incubated in the presence of 100 ng/well IL-6 and 50 ng/well IL-21 for 72 h at 37°C to get Tfh cells (Awe et al, 2015).

Western Blotting

Spleen Tfh cells and joint tissues were directly dissociated with Protein Extraction Kit (CW0891, CWBIO). Protein concentration in the lysates was detected using the BCA Assay Kit (KGP902, Keygen Biotech). After SDS-PAGE separation and transfer to PVDF membranes, the membranes were incubated overnight at 4 °C with following primary antibodies: TLR2 (bs-1019R, Bioss), TLR4 (Santa Cruz), TLR9 (NBP2-24729, Bio-Techne China Co. Ltd.), p-NF-κB p65 (AF2006, Affinity), NF-κB p65 (R&D Systems, Inc.), CXCR5 (49855, SAB), Bcl-6 (H0918, Santa cruz) and ICOS (ab175401, Abcam), GAPDH (ET1601-4, HUABIO). Dilute the above antibodies with antibody diluent (WB100D, New Cell & Molecular Biotech) according to the instructions. The protein expression was detected by ECL luminescent (BMU102-CN, Abbkine).

Total RNA Extraction and Real-Time Fluorescence Quantitative PCR

Use the RNA extraction kit (DP419, TIANGEN) to isolate RNA from spleen lymphocytes, followed reverse transcription into cDNA using reverse transcription kit. The real-time quantitative PCR is run as: 95°C for 30s, PCR reaction: 95°C for 5 s and 60°C for 34s for 40 cycles. Relative quantitation expression of TLR2, TLR4, TLR9, NF-κB p65, p-NF-κB p65, CXCR5, ICOS, Bcl-6 and β-actin mRNA was analyzed using the $2^{-\Delta\Delta Ct}$ method (Table 2). All primers were synthesized by Sangon Biotech (Shanghai).

Immunohistochemical Assay

Paraffin sections were dewaxed, antigen repaired, sealed and stained according to the instructions of the kit. Several fields were randomly selected through the microscope for image acquisition, and the final percentage of positive staining was quantitatively analyzed using Image J.

Statistical Analysis

The data results are shown with mean ± standard deviation (SD). One-way ANOVA was used for comparison between multiple groups, and *T*-test was used for comparison between two groups (GraphPad 8.0). Pearson correlation analysis was used for correlation analysis. Take $P<0.05$ on both sides as the standard with statistically significant difference.

Results

The Subsets of Peripheral Blood CD19+ B Lymphocytes and CD3+CD4+CXCR5+ICOS+ Tfh Cells in Rheumatoid Arthritis Patients Increased

Tfh cells and $CD19^{+}$B lymphocytes in rheumatoid arthritis patients and healthy controls were analyzed by flow cytometry. Our research found that the percentage of B lymphocytes and Tfh cells in rheumatoid arthritis patients

Table 2 Sequences of Primers for RT-qPCR

Mouse-TLR2 Forward	**GACTCTTCACTTAAGCGAGTCT**
Mouse-TLR2 Reverse	AACCTGGCCAAGTTAGTATCTC
Mouse-TLR4 Forward	GCCATCATTATGAGTGCCAATT
Mouse-TLR4 Reverse	AGGGATAAGAACGCTGAGAATT
Mouse-TLR9 Forward	GACTTCAGCGGCAACGGTATGG
Mouse-TLR9 Reverse	TAGTTGTCTCGGAGGCTCAGCAG
Mouse-ICOS Forward	AGGGTGTGCAGCTTTCGTTGTG
Mouse-ICOS Reverse	TGTGTTGACTGCCGCCATGAAC
Mouse-CXCR5 Forward	GAAAACGAAGCGGAAACTAGAG
Mouse-CXCR5 Reverse	AGGAAGATGACAATGTGGTAGG
Mouse-Bcl-6 Forward	AACCATACAAATGTGATCGCTG
Mouse-Bcl-6 Reverse	CACAAATGTTACAGCGATAGGG
MS-ACTB- Forward	GTGCTATGTTGCTCTAGACTTCG
MS-ACTB-Reverse	ATGCCACAGGATTCCATACC

increased significantly, indicating that B lymphocytes and Tfh cells were involved in the pathogenesis of rheumatoid arthritis (Figure 1).

Tfh Cells are Obviously Correlated with Rheumatoid Arthritis Related Inflammatory Factors

In order to understand whether Tfh cells participate in the pathogenesis of rheumatoid arthritis, we analyzed whether Tfh cells are related to clinical parameters and inflammatory factors. Significant correlations were found between the Tfh cells and B lymphocytes (r = 0.4238, $P = 0.0196$) (Figure 2a), RF (r = 0.4468, $P = 0.0133$) (Figure 2b), IL-21 (r = 0.4518, $P = 0.0122$) (Figure 2c), IL-10 (r = −0.4704, $P = 0.0087$) (Figure 2d).

Next, we detected the expression of serum Tfh cell related factors in rheumatoid arthritis patients. ELISA results indicated that serum IL-21 levels in rheumatoid arthritis patients were significantly increased ($P<0.01$) (Figure 2e,). However, serum IL-10 levels decreased significantly ($P<0.01$) (Figure 2f).

Content of Various Alkaloids in Total Alkaloids of Sophora Alopecuroides Linn

The contents of aloperine, sophoridine, oxymatrine, oxysophoridine, matrine and sophoridine in the Total alkaloids of *Sophora alopecuroides* Linn. were 0.1%, 1.0%, 28.9%, 0%, 24.4% and 2.2%. Therefore, we found that matrine and oxymatrine are main active components in Total alkaloids of *Sophora alopecuroides* Linn. (Figure 3).

Total Alkaloids of Sophora alopecuroides Linn. Improves the Joint Swelling Degree, Arthritis Score and Joint Destruction of CIA Mice

In order to know whether Total alkaloids of *Sophora alopecuroides* Linn. can relieve symptoms of rheumatoid arthritis, we established a collagen induced arthritis (CIA) mice model by subcutaneous injection of bovine type II collagen and adjuvant

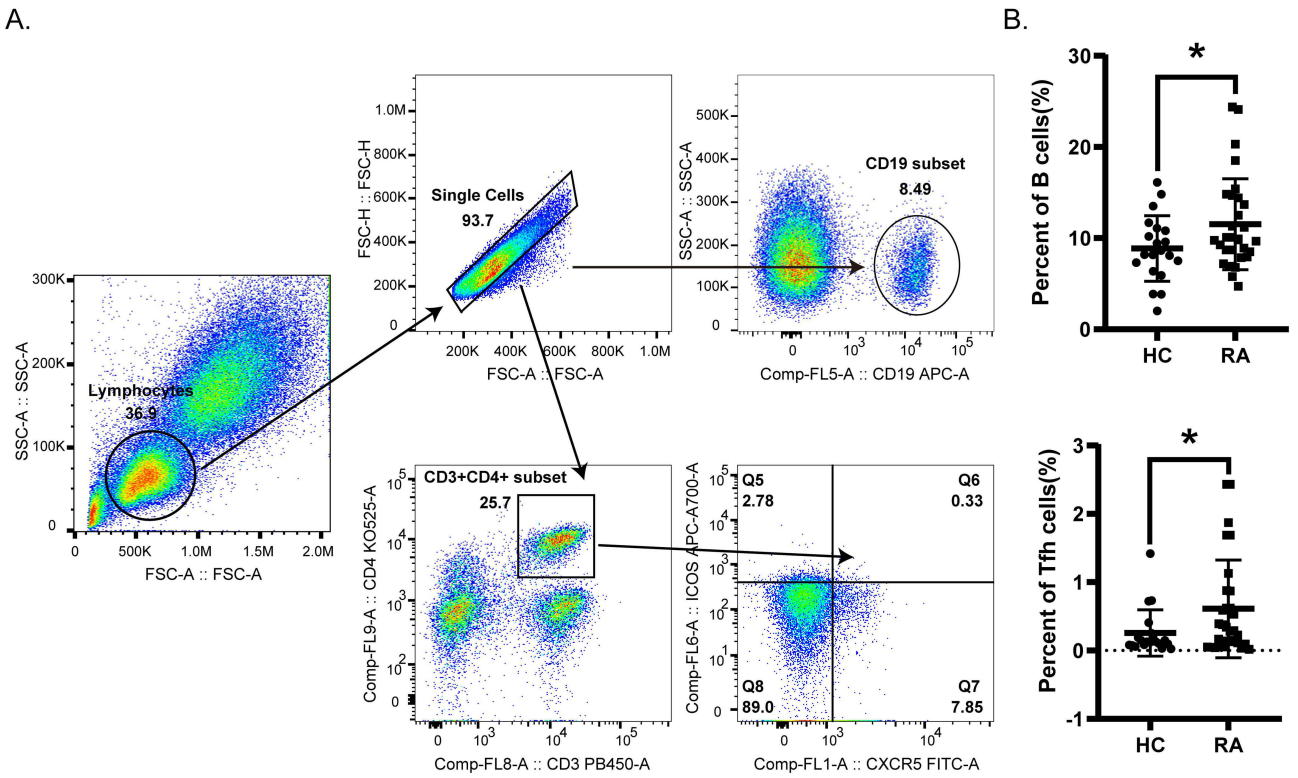


Figure 1 The number of B lymphocytes and Tfh cells in RA patients was detected by flow cytometry. a. Classification strategy and flow chart of Tfh cell and B lymphocytes phenotypes by flow cytometry. Tfh cells ($CD4^+CD3^+ICOS^+CXCR5^+$), B lymphocytes ($CD19^+$). b. The frequency of Tfh cells (*$P<0.05$) and B lymphocytes (*$P<0.05$) in RA patients and healthy controls.

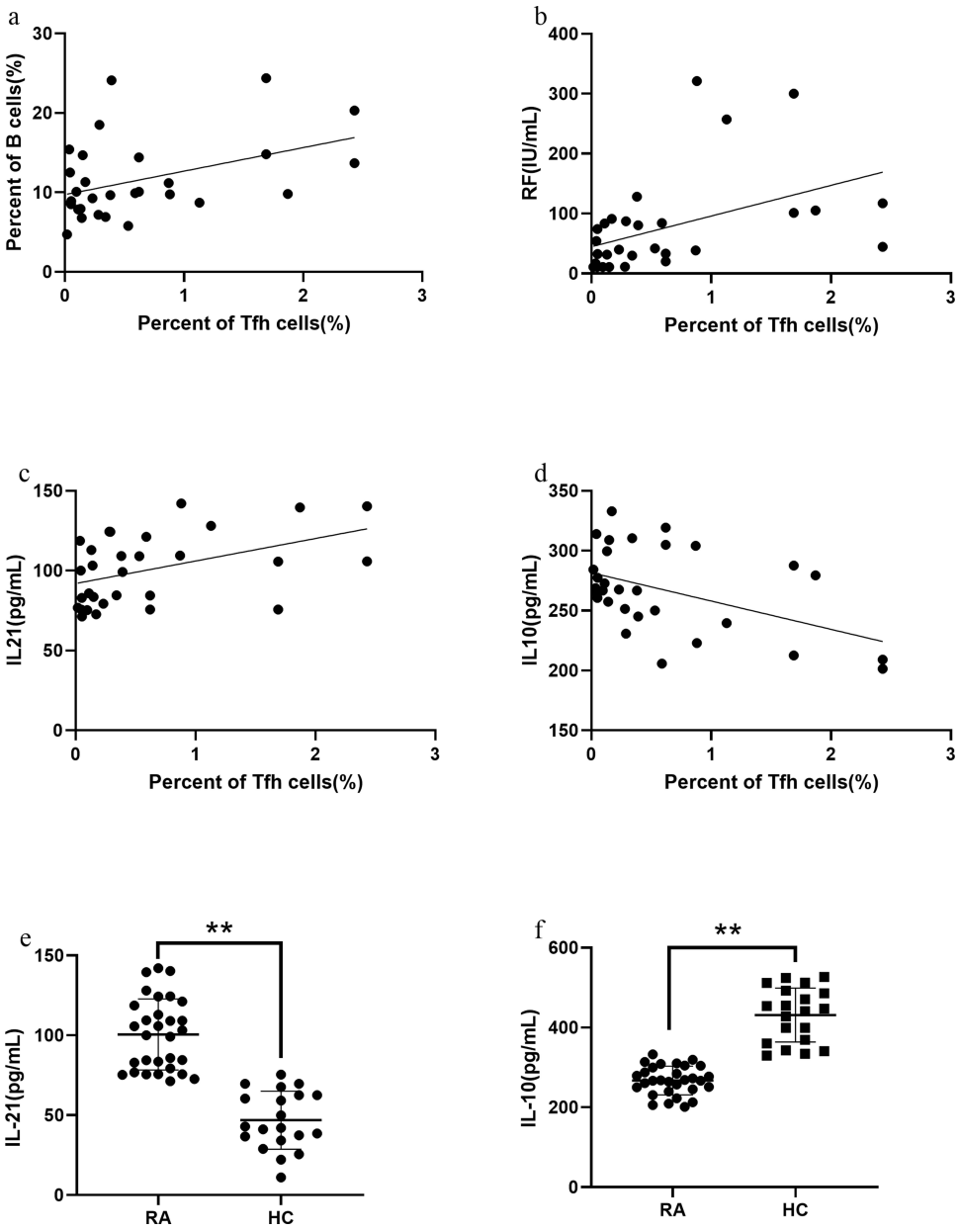
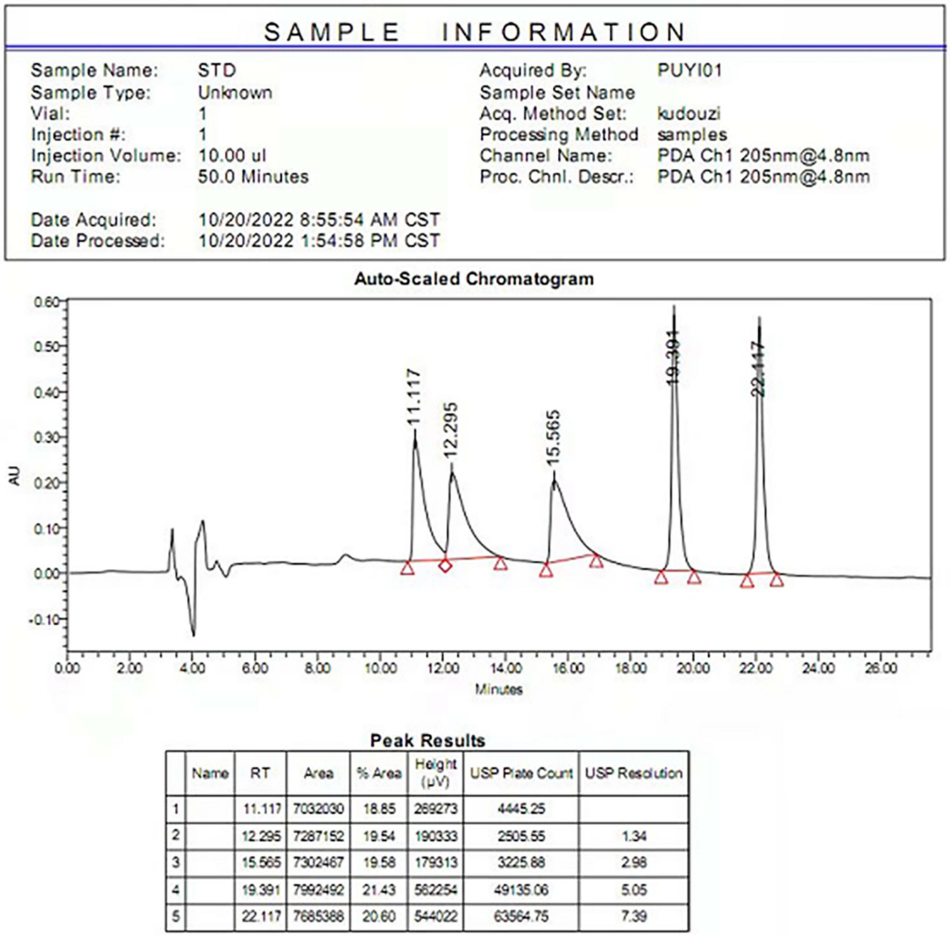


Figure 2 Correlation analysis of inflammatory factors and the percentage of Tfh cells to B lymphocytes in RA patients. (**a**) B lymphocytes (r = 0.4238, *P* = 0.0196). (**b**) RF (r = 0.4468, *P* = 0.0133).(**c**) IL-21 (r = 0.4518, *P* = 0.0122). (**d**) IL-10 (r = −0.4704, *P* = 0.0087). Serum levels of Tfh cell related cytokines IL-21 and IL-10 in healthy controls and RA patients were detected by ELISA. (**e**) Serum IL-21 level. (**f**) Serum IL-10 level. ***P* < 0.01 compared with healthy controls.

into the tail. Our results showed a significant increase in joint swelling and arthritis score in the CIA model group (Figure 4a). Compared with the model group, the joint swelling degree (Figure 4b, Table 3) and arthritis score (Figure 4c, Table 4) of the high dose and medium dose TASA group were reduced ($P<0.05$). HE staining (Figure 4d) showed that synovial hyperplasia was minimal in the high dose and medium dose TASA group, and the surface of articular cartilage was relatively smooth. This indicates that TASA can significantly improve the pathological changes of mouse joint.

Total Alkaloids of Sophora alopecuroides Linn. Significantly Decreases the Percentage of Spleen B Lymphocytes and Tfh Cells in CIA Mice

The spleen lymphocytes of each group were isolated, the molecules of B lymphocytes and Tfh cells were fluorescently labeled, and the change of the proportion of B lymphocytes (Figure 5a and d) and Tfh cells (Figure 5b and c) in the spleen of mice was



	Name	RT	Area	% Area	Height (μV)	USP Plate Count	USP Resolution
1		11.117	7032030	18.85	269273	4445.25	
2		12.295	7287152	19.54	190333	2505.55	1.34
3		15.565	7302467	19.58	179313	3225.88	2.98
4		19.391	7992492	21.43	562254	49135.06	5.05
5		22.117	7685388	20.60	544022	63564.75	7.39

Figure 3 Determination of aloperine, sophoridine, oxymatrine, oxysophoridine, matrine, sophocarpine in total alkaloids of *Sophora alopecuroides* L. by HPLC. (From left to right: 1. Matrine 2. Oxysophoridine 3. Sophoridine 4. Oxymatrine 5. Sophocarpine).

analyzed by FCM. There was a significant reduction in the frequency of the proportion of spleen Tfh cells and B lymphocytes in normal group compared with the CIA model group ($P<0.05$). Compared with the CIA model group, B lymphocytes and Tfh cells in TASA group were reduced ($P<0.05$), suggesting that Total alkaloids of *Sophora alopecuroides* Linn. can down-regulate the ratio of B lymphocytes and Tfh cells in CIA mice, thereby inhibiting the production of auto-antibodies.

Total Alkaloids of Sophora alopecuroides Linn. Represses Serum Inflammatory Factors Levels of IgG1, IL-21 and IgG2a in CIA Mice

We use ELISA kit detects the levels of IgG1, IL-21, IgG2a, and CIIAb in the serum of CIA mice (Figure 6). Compared with the CIA model group, the serum levels of IgG1, IL-21, and IgG2a in the high dose TASA group was reduced ($P<0.05$), but the type II collagen-specific antibody showed no statistical difference between TASA treatment group and the model group ($P> 0.05$). These results showed that the administration of Total alkaloids of *Sophora alopecuroides* Linn. can relieve immune inflammation and symptoms caused by the production of antibodies in CIA mice.

Total Alkaloids of Sophora alopecuroides Linn. Significantly Inhibits the Expression of TLR2, p-NF-κB p65, TLR9, CXCR5, ICOS, and Bcl-6 in the Ankle Joints of CIA Mice

The expressions of TLR2, p-NF-κB p65, TLR9, NF-κB p65, CXCR5, ICOS, and Bcl-6 in the mice joints were analyzed by Western blotting (Figure 7b). The results indicated that, compared with the CIA model group, TLR4, p-NF-κB p65, CXCR5, ICOS, Bcl-6 protein expression reduced in the high dose TASA group ($P<0.05$). At the same time, the

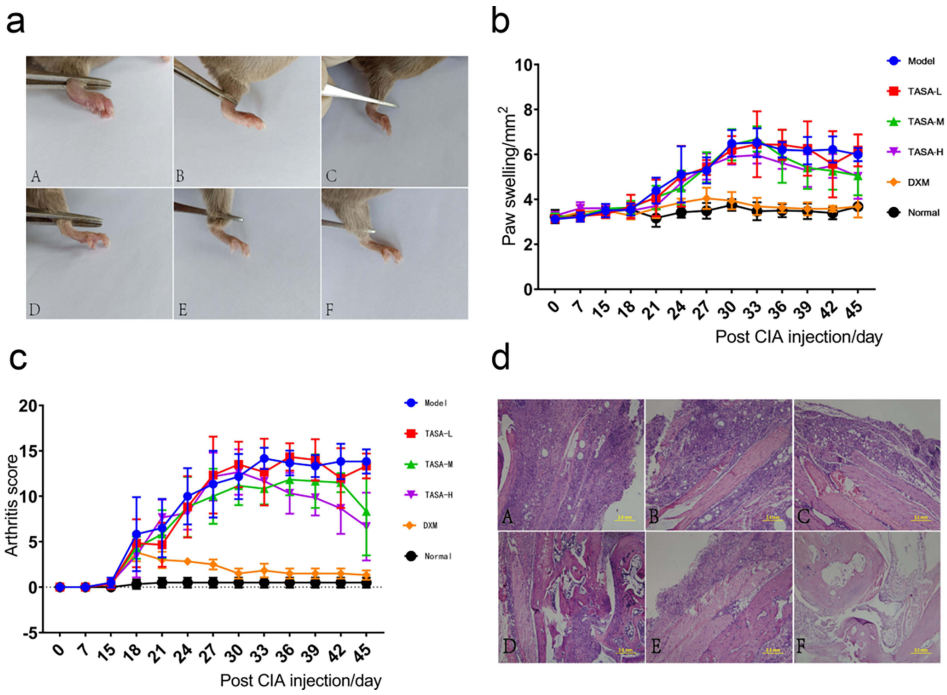


Figure 4 TASA therapy relieve the development of arthritis in CIA mice. (**a**)Representative picture of hind paw (A. Model. B. TASA-L. C. TASA-M. D. TASA-H. E. DXM. F. Normal). (**b**)Hind joint swelling is measured every three days. (**c**)Arthritis score is measured every three days. (**d**) H & E stained pictures of joints (A. Model. B. TASA-L. C. TASA-M. D. TASA-H. E. DXM. F. Normal) (n=6). TASA, Total alkaloids of *Sophora alopecuroides* L. CIA, collagen-induced arthritis. DXM, dexamethasone. TASA-L, Low dose Total alkaloids of *Sophora alopecuroides*. TASA-L, Low dose Total alkaloids of *Sophora alopecuroides*. TASA-M, Medium dose Total alkaloids of *Sophora alopecuroides*. TASA-H, High dose Total alkaloids of *Sophora alopecuroides*.

immunohistochemical results (Figure 8) are consistent with the WB results, meaning that Total alkaloids of *Sophora alopecuroides* Linn. may regulate the number of Tfh cells through the TLRs/NF-κB signaling pathway.

Total Alkaloids of Sophora alopecuroides Linn. Significantly Reduces the Expression of TLR2, TLR4, TLR9, Bcl-6, ICOS, and CXCR5 in Spleen Tfh Cells of CIA Mice

The activation of NF-κB signaling pathway and Tfh cells can lead to excessive production of inflammatory cytokines. To further study the molecular pharmacological mechanism of TASA on CIA mice, we run RT-qPCR (Figure 9) to detect

Table 3 Effect of TASA on the Joint Swelling in CIA Mice $\left(\bar{x} \pm s\right)$

Post CIA injection	Model	TASA-L	TASA-M	TASA-H	DXM	Normal
0	3.120±0.093	3.135±0.160	3.223±0.145	3.275±0.135	3.151±0.108	3.236±0.296
7	3.217±0.203	3.255±0.252	3.224±0.124	3.600±0.274	3.437±0.299	3.289±0.093
15	3.500±0.296	3.345±0.179	3.586±0.211	3.608±0.176	3.509±0.238	3.460±0.231
18	3.544±0.261	3.683±0.519	3.634±0.297	3.494±0.341	3.274±0.165▲	3.541±0.238
21	4.394±0.577	4.066±0.812	4.086±0.522	3.713±0.462*	3.602±0.434*	3.168±0.387*▲
24	5.111±1.253	5.031±1.362	4.510±0.775	4.737±0.330	3.858±0.717*▲	3.427±0.239*▲
27	5.289±0.580	5.418±0.331	5.428±0.668	5.460±0.584	4.045±0.471*▲	3.491±0.350*▲
30	6.489±0.592	6.210±0.607	6.432±0.694	5.902±0.354	3.941±0.383*▲	3.751±0.256*▲
33	6.525±0.640	6.456±1.466	6.688±0.563	5.967±0.371	3.687±0.382*▲	3.471±0.399*▲
36	6.207±0.344	6.432±0.669	5.914±1.180	5.616±0.256▲	3.624±0.210*▲	3.501±0.294*▲
39	6.171±0.610	6.264±1.210	5.413±0.948	5.281±0.727▲	3.578±0.287*▲	3.472±0.345*▲
42	6.222±0.580	5.563±1.475	5.264±0.833*	5.491±0.530	3.584±0.131*▲	3.395±0.276*▲
45	5.995±0.294	6.182±0.710	5.061±0.877*▲	5.018±0.990*▲	3.680±0.490*▲	3.677±0.117*▲

Notes: Data are expressed as mean±SD,*P<0.05, vs the Model group.▲P<0.05 vs the TASA-L group.

Table 4 Effect of TASA on the Arthritis Score in CIA Mice $\left(\bar{x} \pm s\right)$

Post CIA injection	Model	TASA-L	TASA-M	TASA-H	DXM	Normal
0	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
7	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
15	0.500±0.540	0.333±0.516	0.500±0.548	0.500±0.548	0.333±0.516	0.000±0.000
18	4.833±4.070	4.833±2.639	4.333±1.211	3.500±2.429	3.833±0.983	0.333±0.516*▲
21	6.500±3.209	4.667±2.422	5.833±2.639	7.667±1.966▲	3.000±0.894*	0.500±0.548*▲
24	10.000±3.098	8.833±3.371	8.833±3.312	8.167±1.835	2.833±0.408*▲	0.500±0.548*▲
27	11.333±3.670	12.333±4.227	10.000±3.033	12.167±2.639	2.500±0.548*▲	0.500±0.548*▲
30	12.167±2.483	13.500±2.510	11.167±2.137	12.667±2.503	1.500±0.548*▲	0.500±0.548*▲
33	14.167±1.169	12.667±3.670	10.833±1.722*	11.667±2.582*	1.833±0.753*▲	0.500±0.548*▲
36	13.667±1.366	14.333±1.506	11.833±1.722*▲	10.333±2.251*▲	1.500±0.548*▲	0.500±0.548*▲
39	13.333±1.211	14.000±2.280	11.667±2.944▲	9.833±1.941*▲	1.463±0.673*▲	0.500±0.548*▲
42	13.833±1.941	12.000±3.286	11.500±1.049	8.667±2.805*▲	1.486±0.786*▲	0.500±0.548*▲
45	13.833±1.329	13.333±1.366	8.333±4.844*▲	6.667±3.724*▲	1.333±0.516*▲	0.500±0.548*▲

Notes: Data are expressed as mean±SD,*P<0.05, vs the Model group.▲P<0.05 vs the TASA-L group.

TLR2, TLR4, TLR9, CXCR5, ICOS, Bcl-6 mRNA expression in Tfh cells (sorting mouse spleen $CD4^+$ T cells and inducing it differentiate into Tfh cells). The results indicated that the mRNA transcription level of TLR2, TLR4, TLR9, Bcl-6, ICOS, and CXCR5 in the high dose TASA group were lower than the CIA model group. Western blotting analysis (Figure 7a) also showed that TASA treatment can inhibit the protein level of Tfh cell-associated factor in Tfh cells and NF-κB signaling pathway. Thus, the administration of Total alkaloids of *Sophora alopecuroides* Linn. in CIA mice may alleviate arthritis through inhibiting TLRs/NF-κB signal transduction and the activity of Tfh cells.

Discussion

Although more and more studies have demonstrated the value of different $CD4^+$ T cell subgroups in the pathogenesis of rheumatoid arthritis, $ICOS^+CXCR5^+CD4^+$Tfh cells and rheumatoid arthritis are rarely studied. In this paper, our research demonstrated that Tfh cells are associated with inflammatory factors such as rheumatoid factor. Our results show that peripheral blood Tfh cells may participate in the inflammatory reaction of rheumatoid arthritis, and provide value for the treatment and diagnosis of rheumatoid arthritis diseases. In this study, Total alkaloids of *Sophora alopecuroides* Linn. was firstly found to alleviate the joint swelling of CIA mice by inhibiting the frequency and function of Tfh cells to our knowledge. We measured the arthritis index of mice in each group, and detected the cytokines by FCM, Western blotting and other technologies. Our results indicated that Total alkaloids of *Sophora alopecuroides* Linn. may via NF-κB signal pathway reduces the number of Tfh cells and their secretion of cytokine IL-21, thereby reducing the proportion of B cells and inhibiting the production of autoantibodies such as IgG1 and IgG2a, thereby achieving the effect of relieving joint swelling in CIA mice.(Figure 10).

The treatment of rheumatoid arthritis has undergone many explorations in the past few decades, changing from traditional (DMARD) (mainly aimed at pain relief and slow down disease progression)[11] to current molecule targeted biological inhibitors. Although the effect is better, it is expensive and easy to cause bacterial or virus infection. Therefore, the search for new drugs to prevent joint damage, fewer side effects, and low cost has drawn great attention. So traditional Chinese medicine increasingly highlights its unique advantages in the process of treating autoimmune diseases.[12] *Sophora alopecuroides* Linn. are drought-tolerant plants that mainly distribute in northwest China. It contains a large amount of alkaloids (such as Total alkaloids of *Sophora alopecuroides* Linn.) which have important biological activities.[13] A large amount of experiments have proved that Total alkaloids of *Sophora alopecuroides* Linn. has antiviral,[14] efficacy of immune regulation. However, it is unclear how Total alkaloids of *Sophora alopecuroides* Linn. suppresses the occurrence of autoimmune diseases.

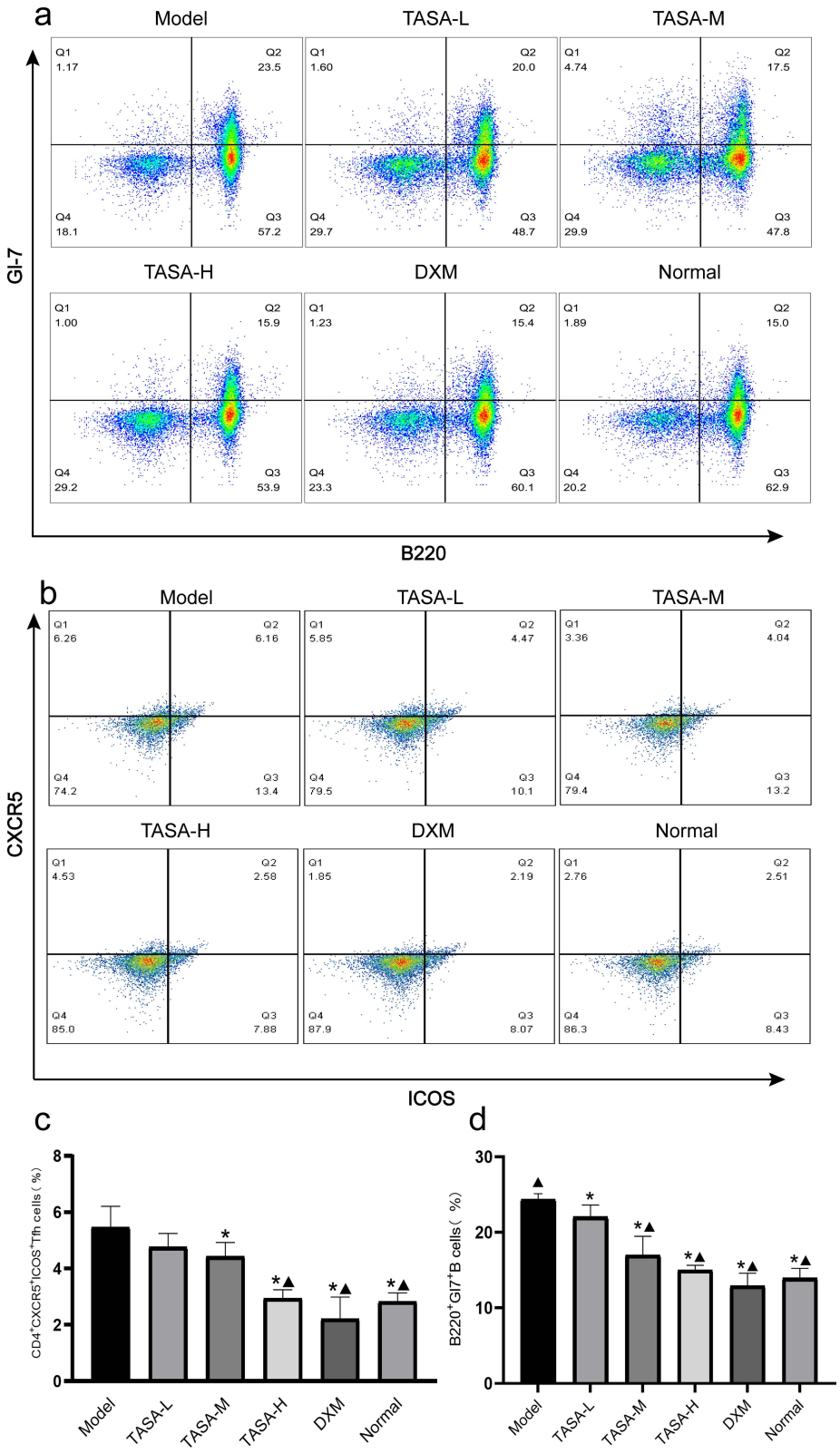


Figure 5 Effects of TASA treatment on Tfh and B lymphocytes in CIA mice. (**a**)Typical FACS plots from the FCM analysis of the $B220^{+}GI\text{-}7^{+}$B lymphocytes obtained from the spleen of CIA mice. (**b**)Typical FACS plots of $CD4^{+}ICOS^{+}CXCR5^{+}$ T cells from the spleen lymphocytes. (**c**)The percentage of Tfh cells ($CD4^{+}ICOS^{+}CXCR5^{+}$). (**d**)The percentage of B lymphocytes ($B220^{+}GI\text{-}7^{+}$) (n=6). Data are expressed use mean ± SD. ▲P<0.05 vs the TASA-L group.*P<0.05 vs the Model group. TASA, Total alkaloids of *Sophora alopecuroides* L. CIA, collagen-induced arthritis. DXM, dexamethasone. TASA-L, Low dose Total alkaloids of *Sophora alopecuroides*. TASA-L, Low dose Total alkaloids of *Sophora alopecuroides*. TASA-M, Medium dose Total alkaloids of *Sophora alopecuroides*. TASA-H, High dose Total alkaloids of *Sophora alopecuroides*.

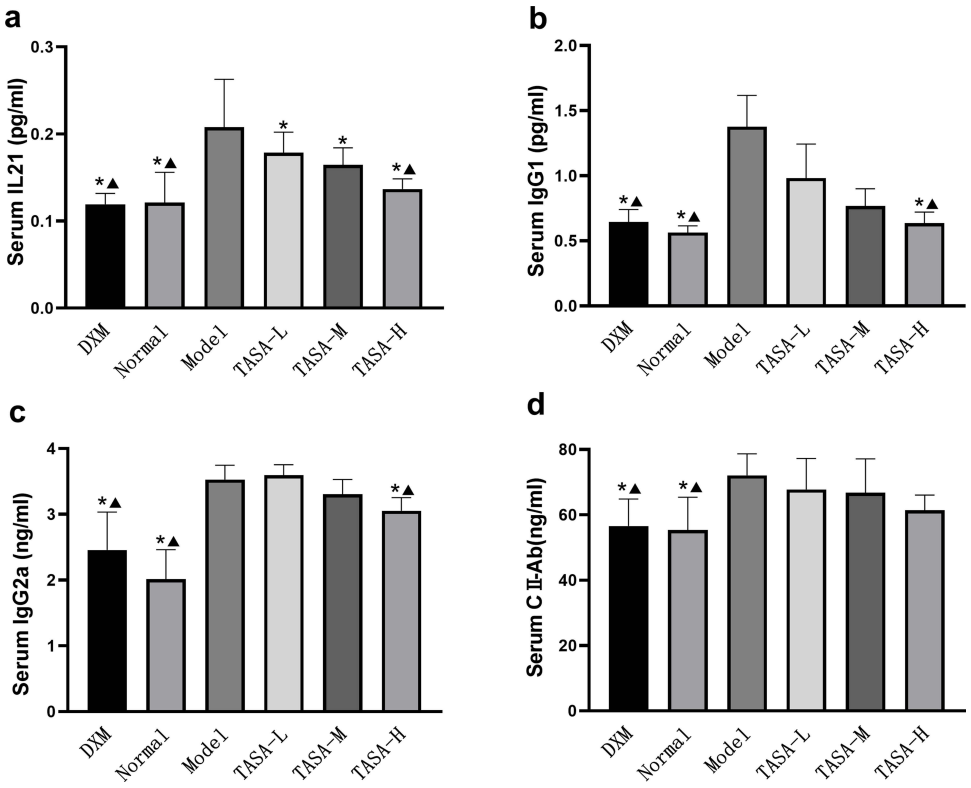


Figure 6 The effects of TASA on serum concentrations of IgG1, IL-21, IgG2a, and CII-Ab. (**a**)IL-21. (**b**) IgG1. (**c**)IgG2a. (**d**)CII-Ab. Levels of IgG1, IL-21, IgG2a were decreased by TASA in a dose-dependent manner (n=6). Data are expressed use mean ± SD. ▲*P*<0.05 vs the TASA-L group. **P*<0.05 vs the Model group.TASA, Total alkaloids of *Sophora alopecuroides* L. CIA, collagen-induced arthritis. DXM, dexamethasone. TASA-L, Low dose Total alkaloids of *Sophora alopecuroides*. TASA-L, Low dose Total alkaloids of *Sophora alopecuroides*. TASA-M, Medium dose Total alkaloids of *Sophora alopecuroides*. TASA-H, High dose Total alkaloids of *Sophora alopecuroides*.

Several studies in related fields clearly demonstrated that Tfh cells increase in a range of autoimmune diseases, such as SLE,[15] type 1 diabetes[16] and rheumatoid arthritis. Our results are consistent with this. We provided evidence that B lymphocytes and Tfh cells were significantly increased in CIA mice, while Total alkaloids of *Sophora alopecuroides* Linn. treatment reduced the number of B lymphocytes and Tfh cells. Indicating that the increase of Tfh cells may be a new feature of rheumatoid arthritis onset. Further implicating that Total alkaloids of *Sophora alopecuroides* Linn. may alleviate the joint swelling by adjusting the Tfh cells.

Tfh cells specifically express ICOS, CXCR5, Bcl-6, and secrete the cytokine IL-21,[17] which plays a major role in Tfh cell differentiation, proliferation, migration and assists B lymphocytes proliferation and differentiation.[18] To further understand the ability of Tfh cells to express IL-21, IL-10, ICOS, CXCR5, Bcl-6, and B lymphocytes to produce autoantibody, we tested the expression of IL-21, ICOS, CXCR5, Bcl-6, IgG1, and IgG2a. As expectation, Total alkaloids of *Sophora alopecuroides* Linn. treatment reduced the level of these factors. These results indicate that TASA may reduce Tfh cell proliferation and differentiation by reducing the transcription factor Bcl-6 of Tfh cells, thereby reducing the differentiation and proliferation of germinal center B lymphocytes, to relieve joint swelling in collagen-induced arthritis mice. However, whether Total alkaloids of *Sophora alopecuroides* Linn. directly regulates the expression of Bcl-6 requires further research.

To make clear how Total alkaloids of *Sophora alopecuroides* Linn. regulates Tfh cells to prevent the development of the collagen-induced arthritis, in the past few years, substantial evidence has revealed that TLRs/NF-κB plays an important role in autoimmune diseases.[19] Abdollahi et al have demonstrated that streptococcal cell wall (SCW) -induced arthritis was significantly reduced in TLR2$^{-/-}$mice.[20] This is due to the reduced of inflammatory cells in the synovium of the joints. To make clear how TASA regulates Tfh cells to prevent the progress of the collagen-induced arthritis, we further examined the effect of TASA on TLRs/NF-κB signaling pathway. We noted that the expression of TLR9, TLR4, TLR2 and its downstream signaling protein p-NF-κBp65 were significantly increased in collagen-induced

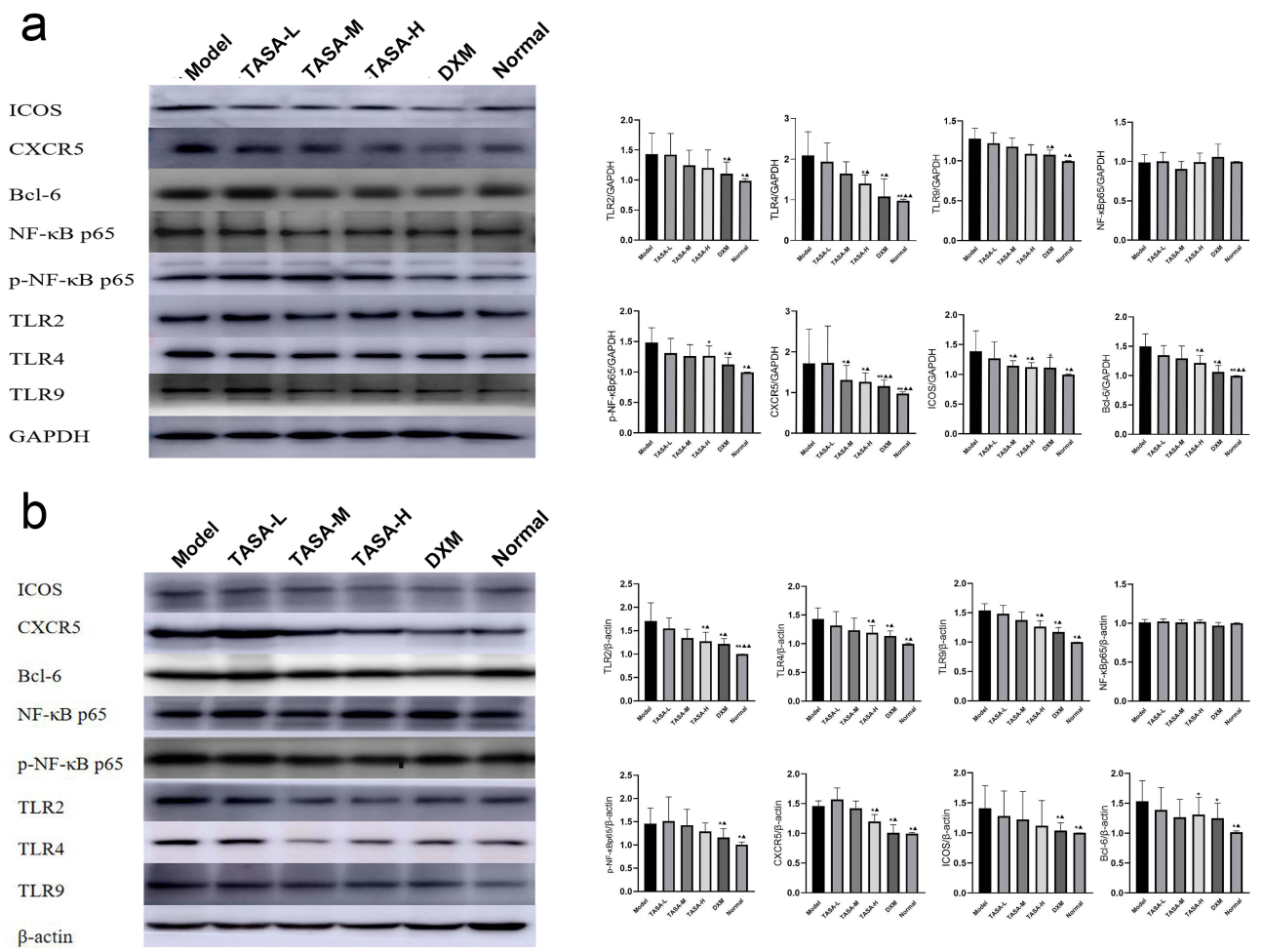


Figure 7 TASA inhibited the protein expression of TLR9, TLR4, TLR2, p-NFκBp65, NF-κBp65, Bcl-6, CXCR5, and ICOS in spleen Tfh cells and ankle joints. (**a**)Examination of TLR2, TLR4, TLR9, NF-κBp65, p-NFκBp65, CXCR5, ICOS, and Bcl-6 in spleen Tfh using Western blotting and quantification by optical density analysis. (**b**)Examination of TLR2, TLR4, TLR9, NF-κBp65, p-NFκBp65, CXCR5, ICOS, and Bcl-6 in ankle joints using Western blotting and quantification by optical density analysis (n=6). Data are expressed use mean ± SD. ▲*P*<0.05, ▲▲*P*<0.01 vs the TASA-L group.**P*<0.05, ***P*<0.01 vs the Model group.TASA, Total alkaloids of *Sophora alopecuroides* L. CIA, collagen-induced arthritis. DXM, dexamethasone. TASA-L, Low dose Total alkaloids of *Sophora alopecuroides*. TASA-M, Medium dose Total alkaloids of *Sophora alopecuroides*. TASA-H, High dose Total alkaloids of *Sophora alopecuroides*.

arthritis mice joint tissue and spleen Tfh cells. But these protein expression was declined in the TASA treatment group, which was accorded with previous reports[21] that the trigger of the canonical NF-κB pathway is vital for Tfh differentiation. This suggested that the TLRs/NF-κB signaling pathway in collagen-induced arthritis mice may be activated and TASA can inhibit TLRs/NF-κB signaling pathway activation in collagen-induced arthritis mice.

The results of this study suggest that Total alkaloids of *Sophora alopecuroides* Linn. may regulate Tfh cells of collagen-induced arthritis mice via the TLRs/NF-κB signaling pathway, thereby inhibit the proliferation and differentiation of germinal center B lymphocytes, reduce the production of autoantibody, and alleviate collagen-induced arthritis in mice.

Our research also has some limitations. The CIA mouse model used in this study, although very similar to the onset of human rheumatoid arthritis, still can not fully stimulate the onset of rheumatoid arthritis. Meanwhile, this study only provides preliminary evidence that Total alkaloids of *Sophora alopecuroides* Linn. has a protective effect on collagen induced arthritis in mice. In future research, we plan to conduct a series of experiments to explore the specific molecular mechanisms of Total alkaloids of *Sophora alopecuroides* Linn. in alleviating collagen induced arthritis in mice.

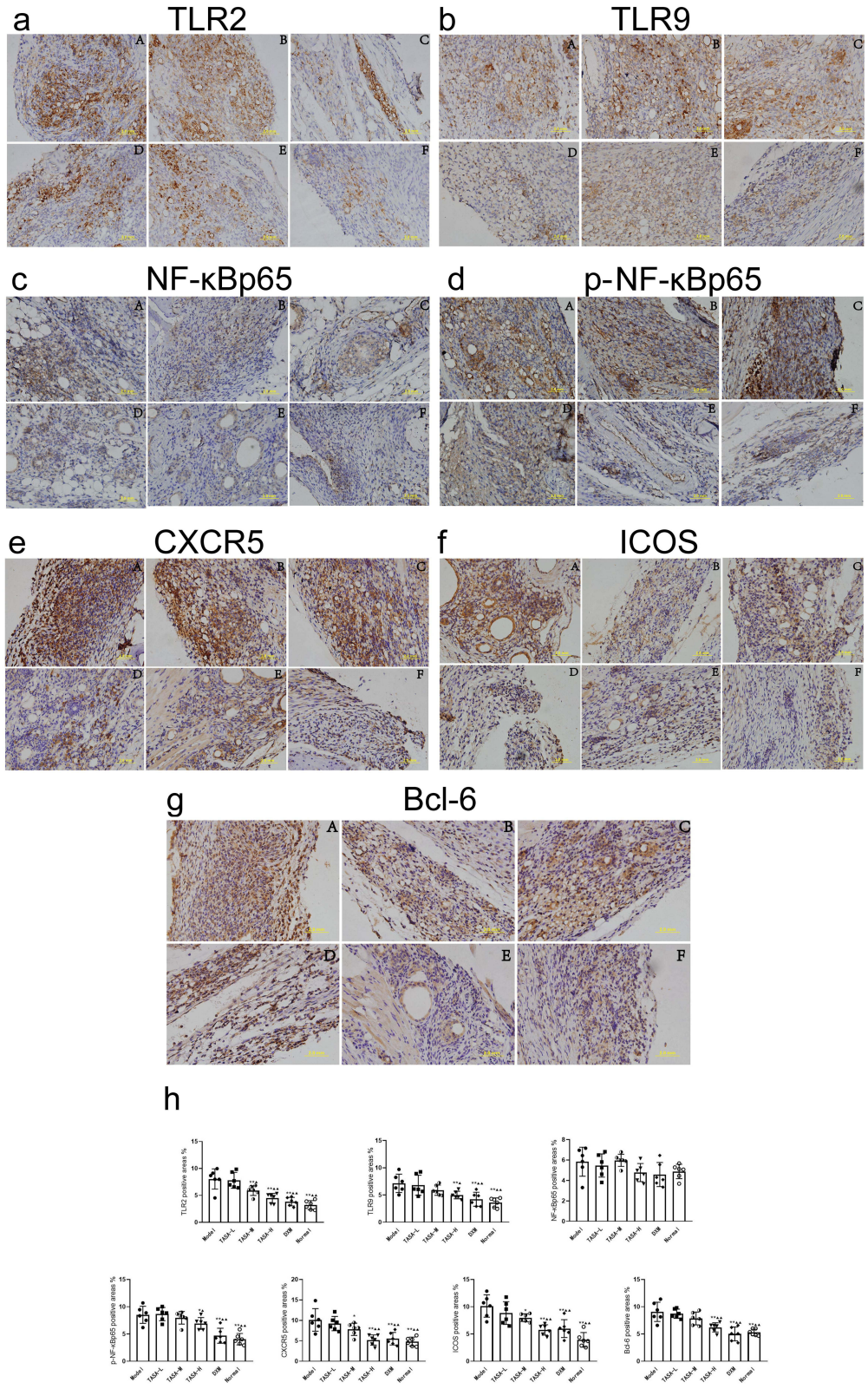


Figure 8 Effects of TASA on the activation of NF-κB-signaling pathways and Tfh cell-associated factor in ankle joints. (**a-g**) Levels of NF-κB-signaling pathways and Tfh cell-associated factor were tested by Immunohistochemistry. (**a**) TLR2. (**b**) TLR9. (**c**)NF-κBp65. (**d**)p-NF-κBp65. (**e**) CXCR5. (**f**)ICOS. (**g**)Bcl-6. (**h**)Quantitation result of immunohistochemistry (n=6). Data are expressed as mean ± SD, ▲P<0.05, ▲▲P<0.01 vs the TASA-L group. *P<0.05, **P<0.01 vs the Model group. TASA, Total alkaloids of *Sophora alopecuroides* L. CIA, collagen-induced arthritis. DXM, dexamethasone. TASA-L, Low dose Total alkaloids of *Sophora alopecuroides*. TASA-M, Medium dose Total alkaloids of *Sophora alopecuroides*. TASA-H, High dose Total alkaloids of *Sophora alopecuroides*. ((**a-g**), (A) Model. B. TASA-L. C TASA-M. D. TASA-H. E. DXM. F. Normal).

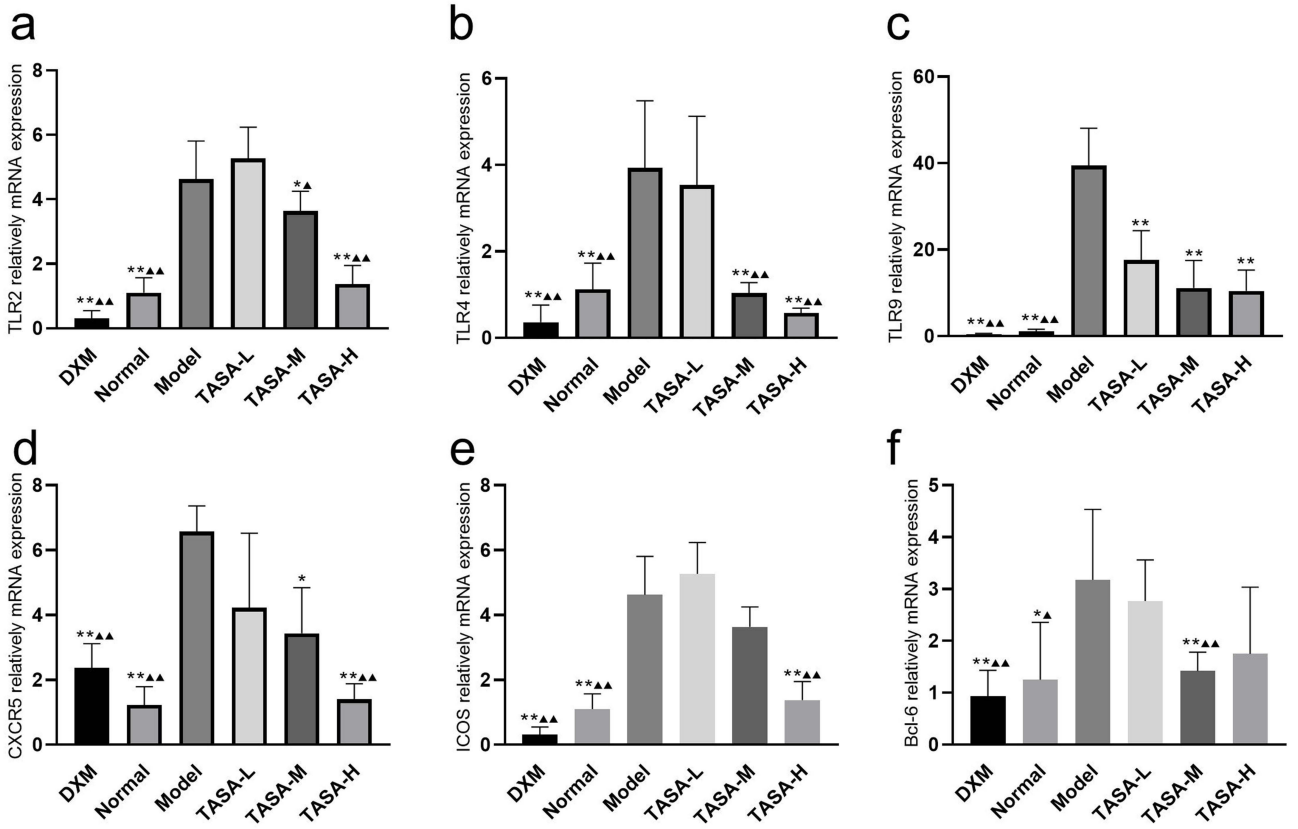


Figure 9 TASA repressed mRNA levels of TLR2, TLR4, TLR9, CXCR5, ICOS, Bcl-6 in spleen Tfh cells. (**a**) TLR2. (**b**) TLR4. (**c**) TLR9. (**d**) CXCR5. (**e**) ICOS. (**f**) Bcl-6. (n=6). Data are expressed as mean ± SD, ▲*P*<0.05, ▲▲*P*<0.01 vs the TASA-L group. **P*<0.05, ***P*<0.01 vs the Model group.TASA, Total alkaloids of *Sophora alopecuroides* L. CIA, collagen-induced arthritis. DXM, dexamethasone. TASA-L, Low dose Total alkaloids of *Sophora alopecuroides*. TASA-M, Medium dose Total alkaloids of *Sophora alopecuroides*. TASA-H, High dose Total alkaloids of *Sophora alopecuroides*.

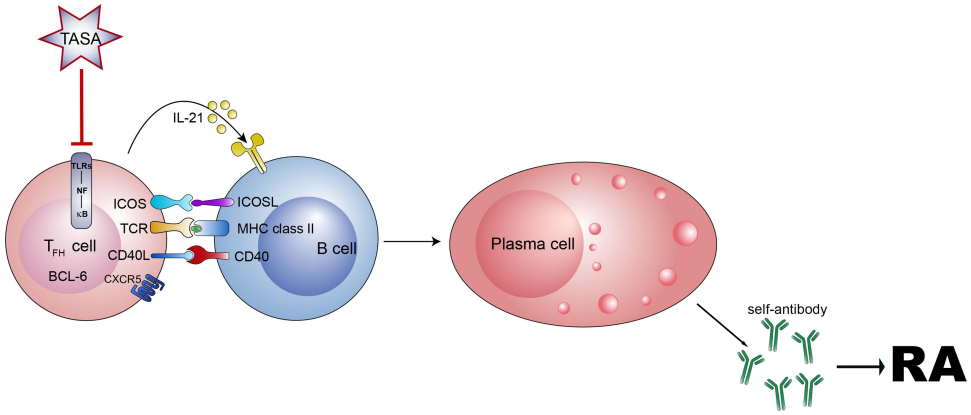


Figure 10 The molecular mechanism of TASA against RA. TASA may regulate the Tfh cells of CIA mice through the TLRs/NF-κB signaling pathway, thereby inhibiting the proliferation and differentiation of germinal center B lymphocytes and reducing the production of autoantibodies to alleviate rheumatoid arthritis.

Conclusion

Taken together, our data showed that the Total alkaloids of *Sophora alopecuroides* Linn. may inhibit TLRs/NF-κB pathway downregulates the number and function of Tfh cells and inhibits GC B lymphocytes, reducing the production of auto-antibodies and alleviating the immuno-pathological process of collagen induced arthritis in mice. Suggesting that, the Total alkaloids of *Sophora alopecuroides* Linn. may be a potential candidate therapeutic medicine of rheumatoid arthritis.

Data Sharing Statement

The data used to support the results of this study is included in this article.

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Disclosure

The authors declare that they have no competing interests in this work.

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