

# Alleviation Effect of Shikonin on Rheumatoid Arthritis in a Collagen-induced Arthritis Murine Model via Induction of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs

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## Abstract

**Objectives:** The process of human immune response is affected by CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs). Reduced suppressive function or the amount of Tregs has been reported in autoimmune disorders. Previous research suggested that shikonin had anti-inflammatory effect on collagen-induced arthritis (CIA). However, whether Tregs are involved in the remission of CIA by the treatment of shikonin remains unclear. The purpose of this study was to explore the intervention effect of shikonin on Tregs in CIA mice.

**Methods:** CIA mice were divided into 5 groups. Group I, Group II and Group III were given shikonin 2 mg/kg, 3.5 mg/kg and shikonin 5 mg/kg, respectively. But Group IV was given meloxicam 50 mg/kg, while Group V was CIA diseased mice. Group VI was normal healthy mice. The expression ratio of Tregs and FoxP3 genes was determined by flow cytometry and qRT-PCR.

**Results:** Our results show that the proportion of Tregs was dramatically reduced in CIA diseased mice, while Tregs significantly increased after shikonin treatment. The proportion of Tregs of 5 mg/kg shikonin group was 5.23 times higher than that of CIA diseased group and FoxP3 gene expression was significantly up-regulated 38.82 times in contrast to the normal healthy group.

**Conclusions:** In brief, shikonin could effectively ameliorate CIA by increasing the proportion of Tregs. The results of this study provide a basis for the change of treatment strategies for human rheumatoid arthritis

**Keywords:** Shikonin; Collagen-induced arthritis; CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells; FoxP3 Gene expression; Correlation.

## 1. Introduction

CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs, which are characterized by constitutionally high expression of IL-2 receptor alpha chain (IL-2R $\alpha$ /CD25), have a negative regulatory effect on T-cell-mediated immune response and inhibit the proliferative activity of T cells with high expression of CD4<sup>+</sup>CD25 [1-3]. High expression of FoxP3 (Fork head box protein 3) detected in cytoplasm is the standard for confirmation of CD4<sup>+</sup>CD25<sup>+</sup>Tregs. Knockout of *FoxP3* gene presents immune dysregulation syndrome and autoimmune disease in human and murine animal, respectively [4]. Secondary metastasis of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs will effectively control clinical symptoms, indicating the close internal

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correlation between FoxP3 and Treg, and its deficiency or dysfunction may lead to susceptible autoimmune diseases [5].

Modern medical research has fully confirmed that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg is involved in the occurrence, development and prognosis of systemic autoimmune diseases [6]. In addition, the reduction of Tregs will aggravate actual clinical manifestations of arthritis. Hand et al. successfully established a mouse arthritis (CIA) animal model by collagen-induced method, and found that the decrease of Treg level would lead to aggravation of the disease, foot swelling and prolonged overall course of disease. Adoptive transfer of Treg will greatly relieve the clinical symptoms and reverse the disease in patients with arthritis [7]. In the acute phase of rheumatoid arthritis (RA), Tregs migrate from the bone marrow and gather around the damaged joints, resulting in a relatively low overall number of Tregs in the bone marrow. Many molecular biological studies have confirmed that FoxP3 expression is absent in CD4<sup>+</sup>CD25<sup>low</sup>FoxP3<sup>+</sup> cells that accumulate near the damaged joints of patients with arthritis, and Th17 cells are hence named, which becomes a key link in the pathophysiological occurrence and development of autoimmune arthritis. The relative decrease of Tregs in the bone marrow of RA patients and the inhibitory effect on T cell activity cannot offset the injury effect of persistent inflammation in the body, thus leading to persistent inflammation [8]. The team led by Avdeeva confirmed that the number of Tregs in the bone marrow showed a trend of regular decrease in the early stage of osteoarthropathies [9]. To sum up, the progress and transformation of RA are closely related to the number and functional changes of Tregs. As the number of Tregs continued to decline, RA became more severe. After introducing CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>Treg into CIA mice by genetic engineering method, the disease was controlled and reversed, indicating that the metastasis of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>Treg to inflammatory lesions is an immune process of self-regulation and repair by the body [10].

Radix arnebiae grows in Yunnan, China, and its dried roots are commonly used in clinical practice. Li et al. 2014 isolated 5 active compounds from *Onosmapaniculatum* Bur. *etFr* root [11]. Shikonin (MW: 288 g/mol), a major active component of *O. paniculatum* Bur. *etFr*, possesses many pharmacological effects, such as sterilization, anti-inflammatory and anti-tumor, promotion of wound healing and regulation of cell and humoral

immunity [12]. In the further established animal experimental model of arthritis, a significant immunosuppressive effect on inflammation was found [13,14]. The application of shikonin can improve the macro score and prognosis of arthritis patients, relieve hand and foot joint swelling, inhibit the further destruction of cartilage, and reduce the serum anti-CII concentration [12,13]. Secondly, significant decreases in tumor necrosis factor- $\alpha$ , Th1 cytokines and interleukin-12 levels were detected in the cases after shikonin intervention, and the levels of IL-10 and IL-4 produced by Th2 cytokines were regulated in CIA mouse models [15]. After the TLR4/MyD88 pathway was blocked, the levels of various inflammatory factors secreted by Th17 cells were significantly reduced, and the immune response process of Tregs was activated [12]. Currently, the key role of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>Treg in the pathogenesis of RA in shikonin therapy of CIA is fully understood, while the mechanism of the peripheral anti-inflammatory effect of this cell population is still unclear. In addition, the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>Treg and *FoxP3* gene activity in CIA patients in various clinical stages after shikonin intervention remain to be further studied. In this study, a CIA mouse model was established, and the expression of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>Treg and *FoxP3* genes changed significantly after shikonin intervention, which explained the molecular mechanism involved in shikonin treatment of CIA to some extent.

## 2. Materials and methods

### Animals, collagen-induced arthritis, and experimental design

Male C57BL/6 mice were provided by Beijing Weituo River Laboratory Animal Technology Co., LTD. This study has passed the evaluation of Animal Experiment Ethics Committee of Kunming Medical University (No.: KMMU2020194), and the administration of mice, specimen extraction and carcasses were performed in accordance with the standards of Animal Care Council of China. All mice were raised in cages and were given free access to adequate water and food. Mice acclimated for 1 week at room temperature of 25°C and humidity of 45 ~ 60%.

Experiments began 9-10 weeks after male C57BL/6 mice had grown up. At the beginning of the study, 100 mol/L bovine collagen was injected intradermally at two sites above the tail of mice on day 0 or normal saline in the control group until day 21. After the animal model was established, the researchers rated the severity of the disease in each

mouse, as follows. 0 point, normal state, no visible lesion of arthritis; 1. Mild redness and swelling on the ankle, and obvious redness and swelling on individual hands or toes; 2. Moderate redness and swelling on ankle joint of the mouse; 3. Severe redness and swelling of the entire paw of the mouse; 4. Redness, swelling and lesions involved in multiple joints. The total severity score of each mouse was added by the sub-points of 4 PAW, and the highest score was 16. The overall incidence of arthritis in mice was assessed at 1 point and the ratio was calculated [13]. The assessment was independently performed by two experienced researchers with no knowledge of the intervention grouping. Serum collagen II concentrations in CIA mice were determined using AN ELISA kit (BD Biosciences, New York, USA).

The CIA mice were randomized into five study groups. On day 21, the CIA-built mice began a daily oral shikonin intervention for seven weeks. The dose of shikonin was 2 mg/kg/day in Group I, 3.5 mg/kg/day in Group II, and 5 mg/kg/day in Group III. Group IV was given 50 mg/kg/day meloxicam intervention treatment, and Group V was set as the control group of CIA disease, and was routinely treated with PBS. The subjects of the sixth group were all healthy mice, which were set as the normal healthy control group. Group V served as a CIA diseased control group receiving phosphate buffer saline (PBS). The sixth group of healthy mice served as the normal healthy control.

#### **Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

The whole blood of 250  $\mu$ l mice from six experimental groups was collected for total RNA extraction. After the collection was completed, the samples were frozen immediately, and then ground thoroughly with liquid nitrogen. The blood samples were homogenized using RNAiso blood kit (TaKaRa, Dalian, China). NanoDrop 2000C spectrophotometer (Thermo Scientific, Loughborough, UK) was used to analyze the concentration and purity of RNA extracted from samples of each research group. Complementary DNA (cDNA) was synthesized using PrimeScript RT kit (TaKaRa, Dalian, China). The cDNA obtained from the above steps was used as a qRT-PCR template for subsequent experiments. Specific primers were designed for mouse *FoxP3* gene (Forward: 5'-CAC CTA TGC CAC CCT TAT CCG-3', Reverse: 5'-CAT GCG AGT AAA CCA ATG GTA GA-3'), and GAPDH was selected as the reference gene (Forward: 5'-TGA CCT CAA CTA CAT GGT CTA CA-3', Reverse: 5'-CTT CCC ATT CTC GGC CTT G-3'). In the

study, SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used in the subsequent QRT-PCR procedure, in which bio-RAD CFX 96 (BIO-rad, CA, USA) was selected as the amplification reagent. QRT-PCR reaction conditions were as follows: 95°C for 30 s, and then 40 PCR cycles (95°C for 5 s, 60°C for 34 s). Finally, fluorescence detection was conducted. The group without cDNA template was used as the negative control group. For all reactions, 10 biological replicates were set for each group, and each biological replicate was repeated for 3 times. Compared with *FoxP3* gene expression of the normal healthy mice, the relative gene expression was calculated with Winer design  $2^{-\Delta\Delta Ct}$  method [16].

#### **Flow cytometric analysis *in vivo***

After the establishment of immune injury, a series of time points were set, and EDTA-K<sub>2</sub> was used to collect peripheral blood of mouse eye keratitis in each experimental group. PBMCs (mononuclear cells) of the samples were isolated on Ficoll solution Ficoll/Hypaque (polysucrose/megluminediphimide, w/w, 2:1) by using density-gradient centrifugation. The whole blood was diluted with Hank's solution at a volume ratio of 1:1. Then, 500 $\mu$ l diluted whole blood was slowly and gently added to the 200  $\mu$ l Ficoll (v/v, 3:1). Samples were centrifuged at 500 g for 20 min and the deceleration of the centrifuge was set as zero. A capillary tube was gently inserted into the grayish white layer of blood cell and PBMCs were sucked out along with the wall of the tube. After the PBMCs were rinsed with cold Hank solution twice, anti-CD4 antibody (labeled with FITC), anti-CD25 antibody (labeled with APC) and isotype control antibody were added. The reaction system was placed in a dark chamber at 4°C, and staining was fully performed for 30 min. The antibody concentration was configured according to the instructions. After dyeing, the reaction system was placed in a centrifuge and centrifuged at 500 g for 10 min. The supernatant in the centrifuge tube was poured out. The precipitation was washed twice again with cold Hank solution. Then, PBMCs were mixed with fixed/osmotic working solution (now used) and incubated in a 4°C thermostatic refrigerator overnight. After Fc blockade was completed, anti-Foxp3 antibody was used for dark staining at room temperature for 45 min. After dyeing, it was washed twice with osmotic buffer. The reaction system was immediately placed on the FACS Calibur (Becton-Dickinson, California, USA) for PBMC testing. WinMDI software (Joseph Trotter, Scripps Research Institute, La Jolla, CA) was used to analyze 10,000 lymphocyte events in each test tube.

### Effect of shikonin on CD4<sup>+</sup> iTreg cells *in vitro*

Naive CD4<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup>CD44<sup>Low</sup> T cells were extracted from healthy C57BL/6 mouse spleen cells and isolated using naive CD4<sup>+</sup> T cell isolation kit (MiltenyiBiotec, Shanghai, China). The cells ( $3 \times 10^6$ ) were cultured in 48 well plates and stimulated with CII (50 µg/ml) in the presence of γ-ray (30Gy) antigen presenting cells (APCs) and 40 unit/ml IL-2 (R & D systems, Beijing, China). The cells were stimulated with CII (50 µg/ml) and TGF-β (R & D) with different concentrations of shikonin, containing 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 nm for 4 days. All cultures were cultured on RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mm HEPES (Invitrogen life technologies, Shanghai, China) and 10% heat inactivated fetal bovine serum (Hyclone, Shanghai, China). The expression of Foxp3 protein was detected by flow cytometry.

#### Statistical analysis

Data on assessment of CIA were analyzed using Paired-Samples T Test. Data on *FoxP3* gene expression and flow cytometric analysis were analyzed using One-Way ANOVA test for multiple comparisons with Post Hoc Tukey's-b and LSD. During data analysis, the collected data of each research group were verified by Shapiro-Wilk and Levene tests to test whether they conformed to the normal distribution and uniformity assumptions. Data analysis was performed using the SPSS 25.0 software package (spssinc., Chicago, USA).  $\alpha < 0.05$  was considered to have a statistically significant difference between the two groups. The linear regression model was analyzed, and the data were imported into SigmaPlot 14.0 (Systat Software Inc., California, USA) to calculate Spearman correlation coefficient. Mean values are standard values, unless otherwise specified.

### 3. Results

#### Effect of shikonin on paw swelling

Figure 1a indicates paw volume change. On day 0, none of the mice showed any lesions such as redness and swelling. Statistical analysis between groups showed significant differences in paw swelling between normal healthy control group and CIA diseased group, although it was invisible to the naked eye. CII immune-treated mice showed significant morbidity, with symptoms such as periarticular erythema and edema in the posterior appearing in the first 14-21 days after immune-treated mice. The normal healthy mice did not exhibit any erythema and oedema. By contrast, the paws of CIA diseased mice continued to become

redder and more swollen until the end of the experiment. The swollen paws of CIA mice were relieved via the administration of meloxicam. It was a remarkable fact that shikonin was just as effective as meloxicam, which inhibited erythema and oedema. Although the degree of paw swelling reached a peak on day 28 in the shikonin treatment group, and then the swelling of the paw gradually was relieved after administration of shikonin, especially treatment with 5 mg/kg shikonin. The paw size of mice in the 5mg/kg shikonin intervention group and normal healthy control group was similar at day 70 (Fig. 1b).

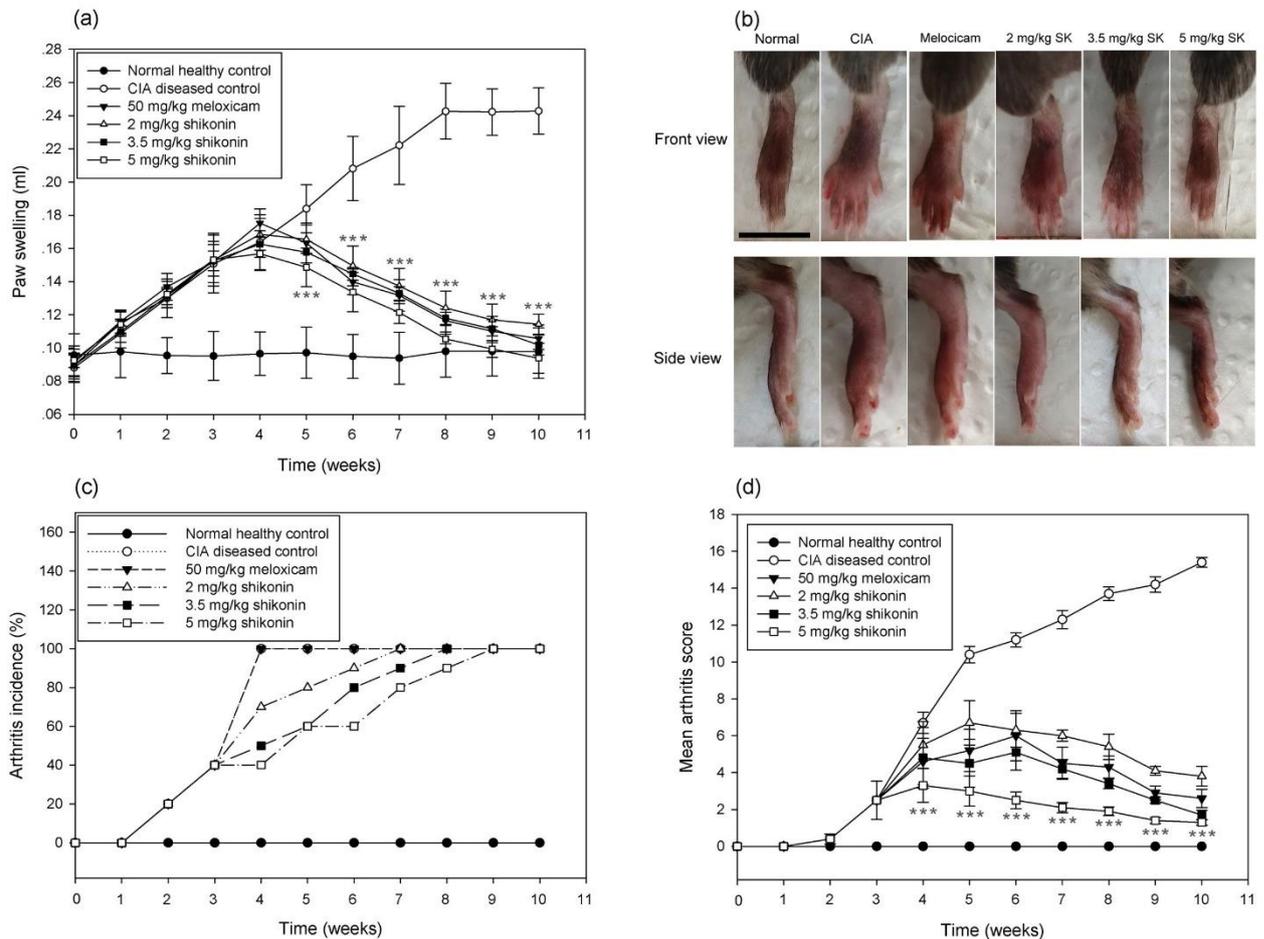
#### Influence of shikonin on incidence and severity of arthritis in CIA mice

To further explore the effect of shikonin on the disease intervention of CIA mice, clinical disease activity scores of each group were assessed weekly from day 0. The results showed that C57BL/6 mice treated by CFA and CII showed significant inflammatory responses in their joints and surrounding areas. After immunization for 14-21 days, the initial stage of arthritis was clearly observed. In each study group, the incidence was 20% on day 14 and 40% on day 21 before shikonin intervention, respectively. After secondary immunization, the incidences of arthritis were rising gradually, reaching 100% in CIA diseased control group on day 28. As shown in Figure 1c, the onset of 100% arthritis in mice in each study group was delayed for 3-5 weeks after the intervention of shikonin. The CIA control group showed clinical symptoms 28 days later. Compared with the meloxicam intervention group, the clinical onset of meloxicam mice was not delayed (Fig. 1c).

Figure 1d shows that the increase in arthritis scores in CII immunized mice over time was time dependent. The CIA disease control mice had a score of 15.4 on day 70. On the day 28 and day 70, compared with the data, the meloxicam intervention group was smaller than the CIA shikonin intervention group ( $p < 0.05$ ). After shikonin intervention, the joint immune injury caused by CII showed significant improvement after 28-70 days ( $P < 0.05$ ). The results fully confirmed that shikonin had good therapeutic effect at various concentrations. Especially at the concentration of 5 mg / kg, the clinical effect was the best ( $0.001 < p < 0.01$ ). 3.5 mg/kg shikonin group was as good as meloxicam treatment group, and 2 mg/kg shikonin group was slightly lower than the meloxicam treatment group ( $p < 0.05$ ), but 2 mg/kg shikonin still had a significant therapeutic effect, compared with CIA diseased

control group ( $0.001 < p < 0.01$ ). It can be seen that the therapeutic effect of shikonin on arthritis is positively correlated with the dose. In particular, the mean arthritis score dropped to 1.3 in 5 mg/kg

shikonin treatment group on day 70. In normal healthy control mice, no lesions were observed in their claws (Fig. 1d).



**Figure 1. (a)** After intervention with shikonin at different doses, changes in the degree of foot swelling of CIA mice were observed (mean  $\pm$  SEM,  $n=10$ ). The asterisk on the error bar indicates a significant difference between the 5mg/kg shikonin treatment group and the CIA disease control group (paired sample T test, \* indicates a significant difference at  $0.01 < p < 0.05$ , \*\* a significant difference at  $0.001 < p < 0.01$ , and \*\*\* a significant difference at  $p < 0.001$ ). **(b)** The anterior and lateral lesions of the hind paw of mice on day 70. Scale = 1 cm. **(c)** The relationship between different doses of shikonin and collagen-induced arthritis in mice. **(d)** Effects of different doses of shikonin on the arthritis score of collagen-induced arthritis mice. The asterisk below the error bar indicates a significant difference between the 5mg/kg shikonin treatment group and the CIA disease control group (paired sample T-test).

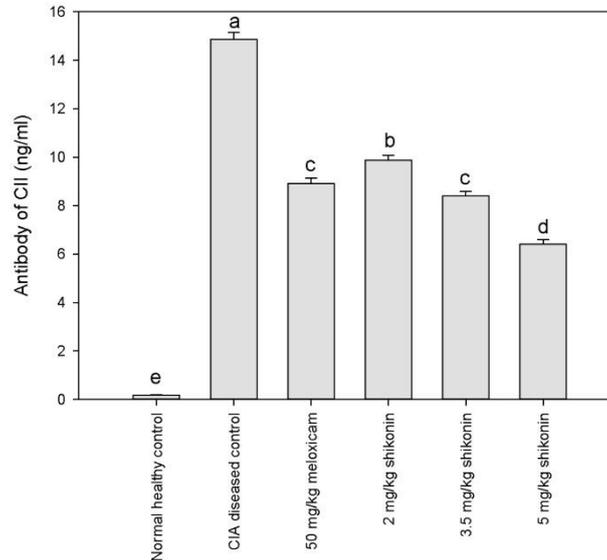
#### Effects of shikonin on the level of anti-CII IgG antibody in CIA mice serum

On day 70, serum levels of anti-CII IgG antibodies were significantly higher in the CIA model group than in the normal healthy control group. After the intervention by meloxicam and shikonin,

the level of anti-CII IgG antibody in serum of mice decreased significantly ( $p < 0.05$ ). Surface data showed that the serum concentration of anti-CII IgG antibody in the 2 mg/kg shikonin intervention group was slightly higher than that in the meloxicam intervention group ( $p < 0.05$ ). Overall, CII antibody

concentrations in serum were significantly higher in the shikonin intervention group than in the CIA disease control group ( $p < 0.001$ ). However, the changes of anti-CII IgG antibody in mice of meloxicam intervention group and 3.5 mg/kg

shikonin intervention group were not obvious. Among them, the most significant change of shikonin intervention resulted in a decrease of serum anti-CII antibody concentration of 5mg/kg in mice (Figure 2)(Table 1).



**Figure 2.** The relationship between shikonin dosage and anti CII IgG antibody in mice serum. The letters in the bar chart showed significant differences among the study groups (one-way ANOVA, Tukey's-b,  $P < 0.05$ ).

**Table 1** Antibody of CII in serum

Group	CII (ng/ml)
Normal healthy control	0.177±0.019 <sup>e</sup>
CIA diseased control	15.253±0.282 <sup>a</sup>
50 mg/kg meloxicam	8.264±0.214 <sup>c</sup>
2 mg/kg shikonin	9.563±0.208 <sup>b</sup>
3.5 mg/kg shikonin	8.355±0.181 <sup>c</sup>
5 mg/kg shikonin	6.782±0.193 <sup>d</sup>

**Table 1.** The changes of anti-CII IgG antibody concentration (means ± SEM, n=10) after 70 days of drug intervention at each dose of shikonin. After the SEM, different letters indicate significant difference between the treatments (One-Way ANOVA Tukey's-b,  $p < 0.05$ ).

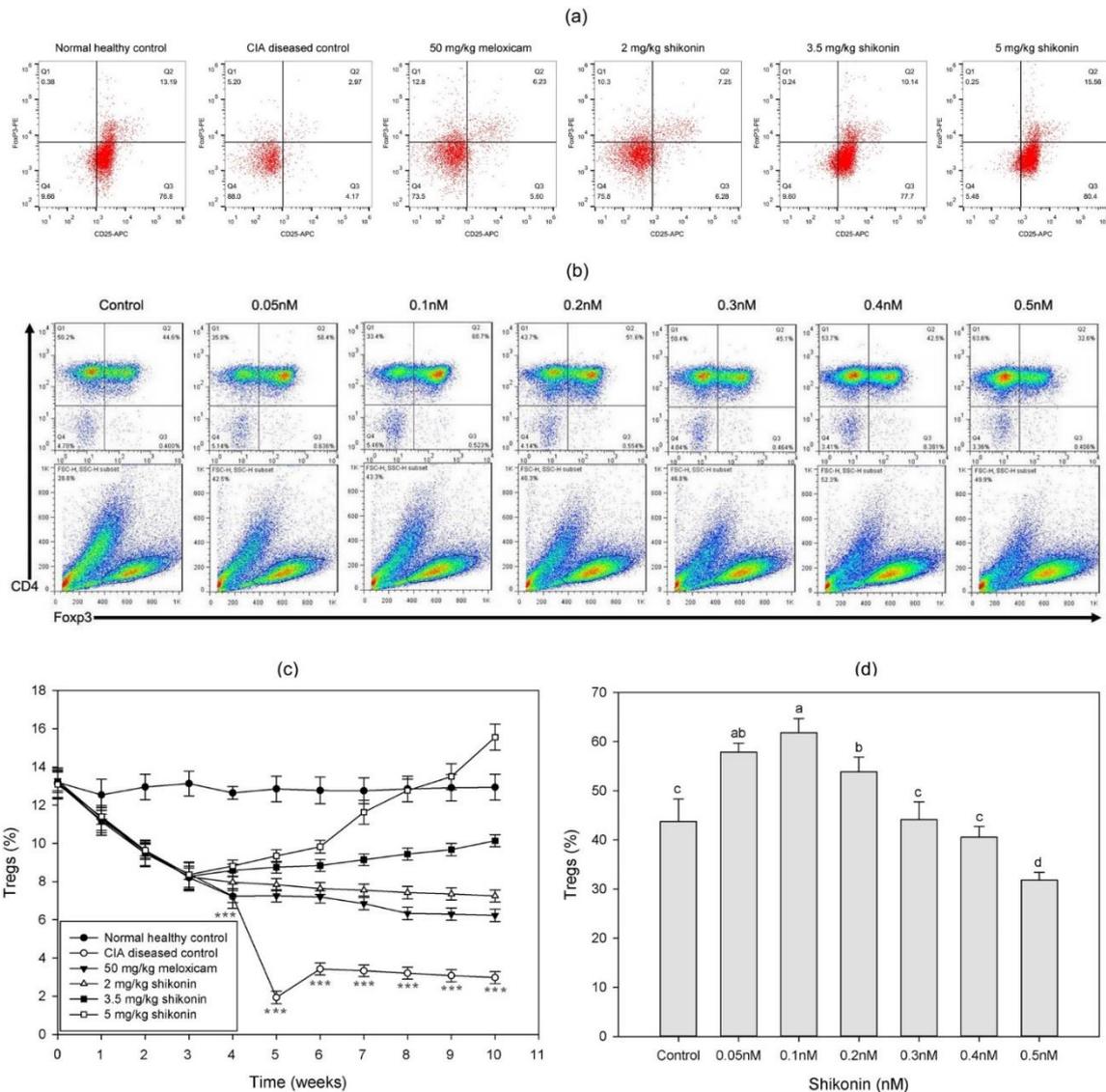
#### Shikonin induction of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs *in vivo* and *in vitro*

Regulatory T cells are of great significance for the maintenance of immune equilibrium and stable state. Based on the surface expression of CD4, CD25 and FoxP3 in these T-cells, the expression levels of these three genes can be measured to analyze the changes in T-cell function. In the study, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg ratio in peripheral blood of the mice was measured weekly. Experimental data showed that the expression ratio of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg in the healthy and normal mice was maintained at 13.191-13.201% ( $p > 0.05$ ).

After CII immunization, the expression of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg in the serum of the mice in the study group showed a significant trend of decrease from day 14 to day 70. Especially the CIA mice with the disease reduced to 2.973% on the 70<sup>th</sup> day (end date). After meloxicam intervention, the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg in serum of mice with CIA was maintained at about 6.229%. However, in the shikonin intervention group, the data showed a significant trend of increase. Especially the serum Tregs concentration of mice in the high-dose shikonin intervention group was significantly higher than that in the low-dose

intervention group, which could increase to 15.56% (Figures 3a and 3c). Shikonin can significantly increase the amount of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg in serum of CIA mice. Whether the amount of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg can also undergo the same change *in vitro* is an aspect of our study. After the intervention by IL-2 and shikonin, CD4<sup>+</sup>CD25<sup>-</sup> T cells from C57BL/6 mice screened by flow cytometry were activated with anti-CD3/CD28 antibody for 4

days, and the number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg was determined again. As shown in Figures 3b and 3d, the number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells *in vitro* also showed a significant increase under the stimulation of 0.05-0.2 nM concentration. In summary, shikonin can significantly increase the number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg both *in vivo* and *in vitro*.

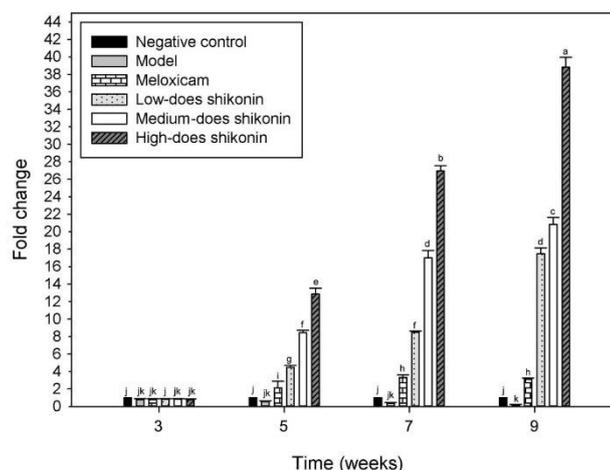


**Figure 3. Shikonin induction of the production of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in CIA mice *in vitro* and *in vivo*. (a) Flow cytometry showed that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs appeared on the 70<sup>th</sup> day. (b) The images of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs were detected by flow cytometry on the 70<sup>th</sup> day of culture *in vitro*. (c) The effect of shikonin concentration on the production of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs *in vivo*. Asterisk indicates that there is a significant difference between the 5 mg / kg shikonin intervention group and the CIA disease group (mean  $\pm$  SEM, n = 10; paired sample t test, \* indicates 0.01 < p < 0.05, \*\* represents significant difference of 0.001 < p < 0.01, \*\*\* = significant difference of p < 0.001). (d) The effect of shikonin concentration on the production of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs *in vitro*. The letters on the bar chart showed significant differences among the treatment groups (one-way ANOVA, Tukey's-b, P < 0.05).**

### Shikonin induction of *FoxP3* gene expression

To further investigate the anti-inflammatory effects of shikonin, the expression of *FoxP3* gene in PBMCs should be examined on the 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup>, and 9<sup>th</sup> weeks by qRT-PCR. As shown in Fig. 4, the expression of *FoxP3* gene in PBMCs was significantly down-regulated with CIA diseased mice, whereas shikonin and meloxicam continuously up-regulated *FoxP3* gene expression levels compared with the CIA diseased group. In week 9, when arthritis was most severe, the degree of swelling in

the soles of the feet was evaluated, and the *FoxP3* gene expression was the lowest in CIA diseased mice. It was 0.19 times lower than that of the normal healthy mice (Fig. 4). However, at the moment, the *FoxP3* gene expression in CIA mice of 5 mg/kg shikonin group was the highest. It was 38.82 times higher than that of the normal healthy mice ( $p < 0.001$ ) (Fig. 4). These findings indicated that shikonin up-regulated gene expressions of *FoxP3* in PBMCs of CIA mice and was indeed anti-inflammatory in arthritis.



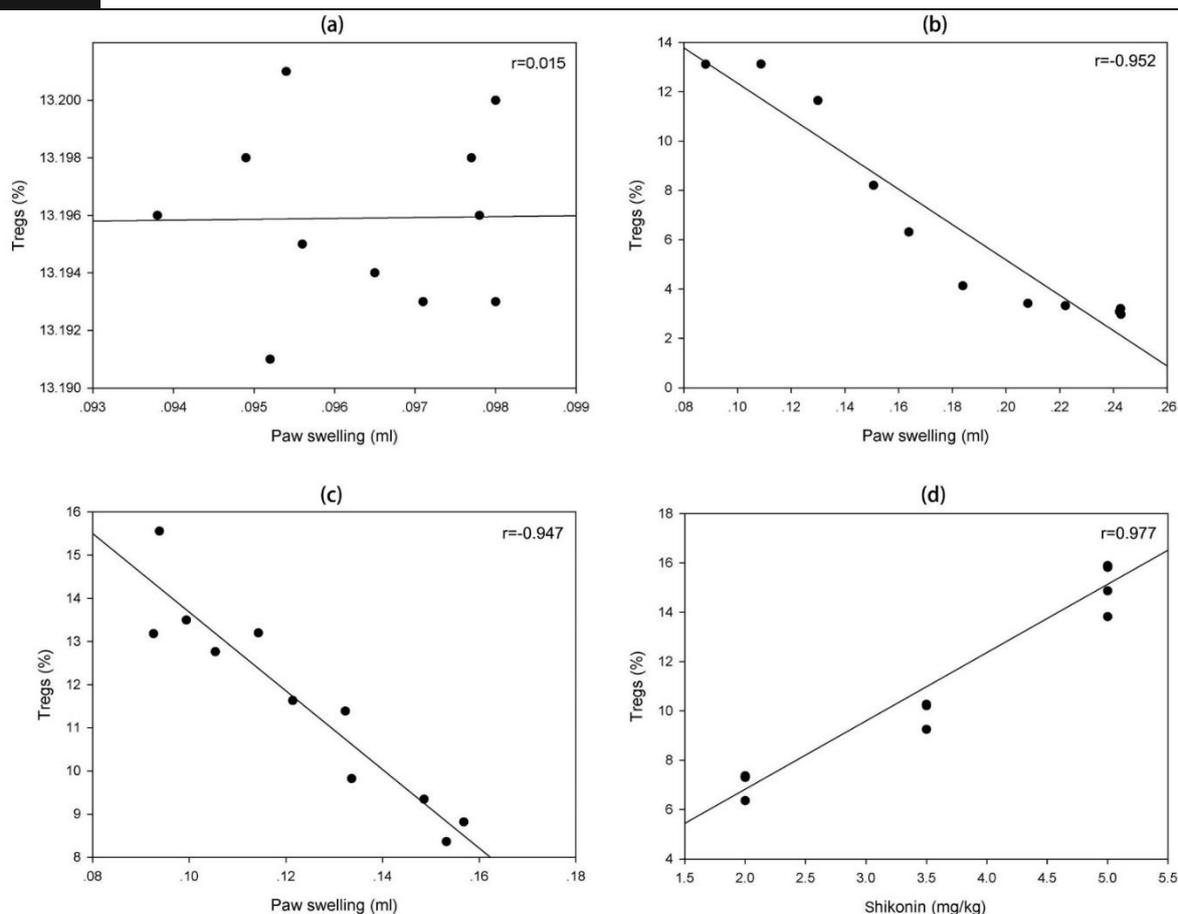
**Figure 4.** The effect of shikonin concentration on *FoxP3* gene expression in CIA mice. The letters on the bar chart showed that there were significant differences in data among different research groups (one-way ANOVA, Tukey's-b,  $P < 0.05$ ).

### Correlation between $CD4^+CD25^+FoxP3^+$ Tregs proportion, paw swelling and shikonin treatment

The results of linear regression analysis were used to determine the efficacy of  $CD4^+CD25^+FoxP3^+$  Treg for shikonin intervention in arthritis. As shown in Figure 5a, changes in the proportion of  $CD4^+CD25^+FoxP3^+$  Treg in serum of normal healthy mice were not directly associated with immune activation. The amount of  $CD4^+CD25^+FoxP3^+$  Treg in the serum of CIA mice was negatively correlated with the degree of foot swelling ( $r = -0.952$ ) (Figure 5b). There was no significant association between the proportion of

$CD4^+CD25^+FoxP3^+$ Treg and foot plantlet swelling after meloxicam intervention or low/medium dose of shikonin intervention. In the high-dose shikonin intervention group, the changes in the proportion of  $CD4^+CD25^+FoxP3^+$  Treg in mice were negatively correlated with the degree of foot plantlet swelling ( $r = -0.947$ ) (Figure 5c). The proportion of  $CD4^+CD25^+FoxP3^+$  Treg was positively correlated with the intervention dose of shikonin ( $r = 0.977$ ) (Figure 5d). Therefore, it can be concluded

that the anti-inflammatory effect of shikonin plays a positive role in quantitative dose-dependent regulation of  $CD4^+CD25^+FoxP3^+$  Treg.



**Figure 5.** The correlation between shikonin and the ratio of  $CD4^+CD25^+Foxp3^+$  Tregs and foot swelling. (a) Correlation between the ratio of  $CD4^+CD25^+Foxp3^+$  Tregs and foot swelling in the normal control group. (b) The relationship between the proportion of  $CD4^+CD25^+Foxp3^+$  Tregs and foot swelling in the CIA control group. (c) Correlation between the ratio of  $CD4^+CD25^+Foxp3^+$  Tregs and foot swelling in the 5mg/kg shikonin group. (d) The effect of shikonin on the ratio of  $CD4^+CD25^+Foxp3^+$  Tregs in vivo.

#### 4. Discussion

CIA is a kind of autoimmune mediated polyarthritis, which has many similarities with human RA in terms of immunity [17].  $CD4^+CD25^+FoxP3^+$  Tregs are one special type of T cells, which can inhibit its own reactivity and directly participate in the peripheral immune tolerance process of the body [1]. In case of autoimmune disorder,  $CD4^+CD25^+FoxP3^+$  Tregs differentiation is reduced, leading to autoimmune diseases, such as RA. Many studies have shown an intrinsic connection between the expression ratio of  $CD4^+CD25^+FoxP3^+$  Treg and disease development in CIA mice [18]. Other studies have shown the effect of shikonin on articular cartilage repair in RA mice [13]. However, there was no correlation between serum  $CD4^+CD25^+FoxP3^+$  Treg and shikonin intervention dose in CIA disease remission. In this

study, a mouse RA model was established to analyze the therapeutic effect of shikonin on this disease. In the treatment of CIA mice, the intervention concentration of shikonin was divided into three gradients, with meloxicam as the positive control group. It was found that shikonin could significantly alter collagen to affect the changes in arthritis. Shikonin could also increase the serum proportion of  $CD4^+CD25^+FoxP3^+$  Treg, affecting the expression of *FoxP3* gene and thus affecting the disease progression of RA mice. Therefore, it can be speculated that in the development of RA mice, autoimmune inflammation is the key to its progression, and the proportion of  $CD4^+CD25^+FoxP3^+$  Treg and the expression of *FoxP3* gene are also decreased. The increased proportion of  $CD4^+CD25^+FoxP3^+$  Treg in serum of CIA mice after shikonin intervention may be directly related to the improvement of inflammatory response.

The changes in RA's condition are directly related to changes in the function and number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs. Some studies have also shown that the relationship between changes in the number of Tregs in the blood of RA patients and their condition score is unclear, which may be directly related to the stage of RA's condition. Therefore, it is very necessary to study the effect of shikonin on the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg in the blood of CIA mice in different disease stages. In the progress of RA, the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg in peripheral blood of animal models showed a downward trend. After shikonin intervention, the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg gradually approached that of healthy mice. It can be speculated that shikonin intervention can affect the TLR/MyD88 pathway, directly inhibit the expression of Th17 cytokines and mediate the immune process of Treg cells [12]. From other perspectives, the high-dose shikonin treatment group was even higher than the normal healthy group at the end of this study (on day 70), suggesting that shikonin was more effective in inducing CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs with the increase in the dose of shikonin. Thirdly, we used cell culture to test the induction effect of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs by shikonin *in vitro*. Cells *in vitro* were more sensitive than cells *in vivo*. Compared with the control group (without shikonin), shikonin at 0.05nM could induce an increase in the number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs, and the induction effect at 0.1nM was the best. When the concentration of shikonin was higher than 0.1nM, the induction effect gradually decreased. When the concentration of shikonin reached 0.5nM, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs were inhibited. This may be due to the toxic effect of high concentrations of shikonin on cultured cells *in vitro*. Finally, from the transcriptome respect, the higher the shikonin concentration, the longer the shikonin treatment time. Shikonin could up-regulate *FoxP3* gene expression in CIA mice, which was the same as shikonin significantly increased the expression of *FoxP3* gene expression in the skin of the psoriasis mice, thus controlling the differentiation of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs by inhibiting AKT/mTOR Pathway [19].

The effect of shikonin on the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in the serum of mice was dose-dependent, which was verified in the study. Research data showed that the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg in serum was significantly negatively correlated with the foot swelling of CIA mice ( $r=-0.955$ ,  $p < 0.001$ ). Pathological analysis showed that the degree of arthritic foot lesions in

mice was closely correlated with the decrease in the number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg. The study data also found that the changes in the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg and the degree of foot metatarsophalangeal swelling lesions were not significant in the serum concentrations of healthy mice, shikonin, low-dose intervention group and meloxicam intervention group. Only after the intervention of high-dose shikonin did the foot swelling and disease release of CIA mice show significant reversal, accompanied by negative correlation ( $r=-0.947$ ), suggesting that high dose of shikonin induces immune activation in CIA mice. After the high-dose shikonin intervention, the proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg in serum was significantly increased, and the redness and swelling volume of toes was also reduced. The degree of paw swelling was alleviated by high-dose shikonin. The change of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in serum of mice was positively correlated with the change of shikonin concentration ( $r=0.977$ ). This research result is consistent with the expectation before the beginning of this study and with many current research results. The results fully confirmed that the mechanism of anti-inflammatory drug therapy for arthritis lies in regulating the function and proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg *in vivo*. [20,21].

The results of this study confirmed that the mechanism of shikonin therapy for CIA involved the changes in the function and proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg. After shikonin intervention, the serum ratio of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg was significantly increased, which improved the immune tolerance of mice. This conclusion is consistent with pharmacological research inference, but it is still limited to sample size, research depth and other reasons difficult to be fully recognized by the academic community. Future studies must further investigate the effect of shikonin on the inflammatory process in RA patients in different pathological stages. In summary, shikonin inhibits CIA by upregulating the expression of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs and *FoxP3* genes, which will be widely used as a new immunomodulatory drug in the clinical treatment of RA.

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**Ethical approval:** This study was reviewed and approved by the Ethics Committee for Experimental Animals of Kunming Medical University, and all mice were treated according to the guidelines of principles of laboratory animal care (NIH publication No. 86-23, revised 1985).

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