Zaocys type II collagen regulates the balance of Treg/Th17 cells in mice with collagen-induced arthritis

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Abstract

Objective

Zaocys type II collagen is an active collagen extracted from Zaocys that has been used to treat rheumatoid arthritis in China for over 1000 years. However, the mechanism still remains unknown. Therefore, we set out to investigate the inhibitory effect and possible mechanism of action of zaocys type II collagen on collagen-induced arthritis.

Methods

Collagen-induced arthritis was induced in C57BL/6 mice by immunisation with type II collagen. After immunisation, the mice were treated with Zaocys type II collagen. Clinical and histological scores were assessed and the cytokine levels in the serum and lymphocytes supernatant from the spleen and mesenteric lymph node were determined by enzyme-linked immune sorbent assay. The T-helper 17 cell and regulatory-T cell frequencies were analysed by flow cytometry and the expression of interest markers was examined by direct immuno-fluorescence.

Results

The arthritis score and severity of histological inflammation and cartilage destruction were dose-dependently reduced after treatment. The analysis results indicated that Zaocys type II collagen significantly increased the proportion of regulatory-T cells and lowered the T-helper 17 cells, it also increased the number of regulatory-T cells and conversely decreased the T-helper 17 cells in synovial tissue compared with the model group. Treatment also caused a higher level of transforming growth factor-β and a decreased production of interleukin -17A.

Conclusion

The oral administration of Zaocys type II collagen potently suppressed the severity of collagen-induced arthritis by repairing the imbalance between regulatory-T cells and T-helper 17 cells, suggesting that it might be a promising candidate for the treatment of rheumatoid arthritis.

Key words

type II collagen, rheumatoid arthritis, collagen-induced arthritis, regulatory-T cell, T-helper 17 cell

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H. Wang conceived and designed the experiments, participated in all the experiments.
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X. Wu participated in the induction of animal models.
X. Chen provided reagents and technical support. J. Li conceived the study, and participated in its design.

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Introduction

Rheumatoid arthritis (RA) is a progressive inflammatory autoimmune disease characterised by synovial inflammation that causes cartilage destruction and subsequent bone erosion (1, 2). Although the exact aetiology of RA remains unclear (2, 3), type II collagen (CII) is an acknowledged auto-antigen which is believed to be responsible for the induction of RA (4). Previous work has indicated that the pathophysiological changes of RA are caused by the combined action of T cells, B cells and pro-inflammatory cytokines (5, 6).

It is indicated that the dysfunction of T cells and the persistence of inflammatory factors are the key factors in inducing and maintaining RA (7). The hyperactivity and the elevated population of Th17 cell is believed to be involved in the formation and development of RA (8), which is linked to the hyper-production of IL-17A secreted by Th17 cells (9-11). Moreover, the Treg cell has also been considered as a new topic of interest patients with RA recently (12). Some studies have revealed that RA patients have lower number of Treg cells in their serum than the healthy (13), demonstrating that the reduction and hypo-function of Treg cells is important in the immune imbalance that causes RA (14). Treg cells, which comprise 5–10% of the $CD4^+$ T cells populations, are identified by the expression of the forehead box P3 protein (Foxp3) (15). And Treg cells suppress immune responses through the release of cytokines such as growth factor-β (TGF-β) and IL-10, in addition to upregulating the expression of suppressive immune-cell surface molecules and inhibiting the expression of genes that activate T cells (16).

What is more, a transformation between Treg and Th17 cell can occur under certain circumstances. TGF-β is a critical differentiation factor for the generation of Treg cells. Studies have shown that naïve $CD4^+$T cells can differentiate into Treg cells with the existence of TGF-β (17, 18). In contrast, expressing TGF-β together with IL-6 can induce the expression of RAR-related orphan receptor gamma t (RORγt), which promotes the differentiation of naïve $CD4^+$T cells into Th17 cells (19-21). Although the limited data available cannot yet prove the important roles that Th17 and Treg cells and their related cytokines play in human arthritic diseases (17, 22), Treg-mediated suppression of arthritic responses in animal models of arthritis makes these cells promising candidates for therapeutic targets in arthritic diseases such as RA. In brief, Treg and Th17 cells are cells with antagonistic functions and a link in differentiation, and the balance between them is crucial for immune homeostasis, as these two cells might be key effective factors in RA.

Zaocys dhumnades (Cantor) (Serpentiformes: Colubridae) are large non-toxic snakes particular in China, which are located in south region of China's Huaihe River and Qinling Mountains. And Zaocys has been widely used to treat RA in China for over 1000 years, of which the active components and their targets are still unclear. According to modern pharmacology studies, zaocys has anti-inflammatory and analgesic effects. The clinic remission rate with the Disease Active Score (DAS28) score ≤2.6 of Zaocys-based traditional Chinese medicine compound therapy in rheumatoid arthritis were up to 80% (23-25). The total effective rate of Zaocys hydrolysates therapy in rheumatoid arthritis were 62.4% (26). *In vivo* experiment certified that Zaocys hydrolysates can reduce the incidence of arthritis and improve the symptoms by suppressing the activity and levels of inflammatory factors TNF-α, IL-1and IL-6 produced by serum in AA rats (27). Our previous study has shown that total protein from zaocys inhibits the proliferation of fibroblast-like synoviocytes and the expressions of TNF-α, IL-1β, and IL-10 in humans (28). Additionally, oral administration of Zaocys type II collagen (ZCII) can ameliorate the clinical signs of arthritis and effectively suppressed the activity and levels of TNF-α and IL-1β produced by synoviocytes in rats with AA(29). However, the mechanism of action exerted by ZCII in collagen-induced arthritis (CIA) mice has not been extensively studied.

This study analysed the changes in Treg

cells, Th17 cells and their cytokines in CIA mice to further understand how ZCII functions in RA, investigate the mechanisms of action displayed by ZCII in CIA mice and definitively identify the target of this treatment.

Materials and methods

Animals

Zaocys and male C57BL/6 mice (aged 6–8 weeks, 25±5g) were provided by Southern Medical University Laboratory Animal Center. The animals were housed under specific pathogen-free (SPF) conditions and fed standard rodent chow and water ad libitum. The studies were approved by the Animal Care and Use Committee of Southern Medical University.

Reagents

The Chicken CII (CCII), Complete Freund's adjuvant (CFA), and red blood cell lysis buffer (RCLB) were purchased from Sigma (USA); the RPMI-1640 and fetal bovine serum (FBS) were from Gibco (USA); the Treg cell flow cytometry detection kit, anti-Mouse CD4 FITC, and anti-Mouse IL-17A PE were purchased from eBioscience (USA); the PMA/Ionomycin mixture, BFA/Monensin mixture, FIX&PERM Kit, Mouse IL-17A Platinum ELISA, and Mouse TGF-beta1 Platinum ELISA were purchased from Liankebio (China).

Purification and identification of Zaocys type II collagen

Type II collagen was extracted and purified from Zaocys by pepsin digestion following the instructions. The molecular weight of ZCII was detected by SDS-PAGE analysis with standard CCII. The ultraviolet absorption of ZCII was detected with an ultraviolet spectrophotometer, with CCII as well. The homology of ZCII and other species was analysed using mass spectrometry (MS) according to the following protocol: The specific target band was excised from the gel and digested by enzymolysis. After purification and concentration with a ZipTip®C18, the sample was detected by MS and its peptide mass fingerprinting (PMF) was achieved. Mascot software was used for the retrieval and to compare the PMF of ZCII in the database. The Swissprot database was searched within the MS and MS/MS models. The animal database was set, and the molecular weight limits were set at 800 and 4000 KD. The proper deviation was 50ppm. At least 4 peptides should be matched between the PMF and the database to meet the filter requirements.

Induction of collagen-induced arthritis and assessment of mouse model

CCII was dissolved in 0.1mol·L^{-1} acetic acid by stirring overnight at 4°C till the final concentration was 4mg • ml^{-1}.The CCII solution was mixed with an equal volume of CFA in an ice-bath to make the emulsion. One hundred microliters (100 μl) of the emulsion was injected into the mice at the base of the tail for immunisation. A booster immunisation was given 7 days after the primary injection. Meanwhile, the other groups received 100 μl-injection of 0.1mol·L^{-1} acetic acid at the same location. The severity of arthritis was assessed by 2 independent observers who evaluate clinical symptoms on a five-point scale: 0=no swelling and focal redness, 1=swelling of finger joints, 2=mild swelling of ankle or wrist joints, 3=severe inflammation of the entire paw, and 4=deformity or ankyloses (30, 31).

Treatment protocols

The CIA mice were divided into 4 groups randomly: a CIA model group, and three ZCII groups. ZCII (10, 20, and40 μg·kg^{-1}·d^{-1}) was intragastrically administered to the mice on days 21 to 28 after immunisation in ZCII groups, while the mice of normal and model groups received an equal volume of acetic acid.

Isolation of lymphocytes from spleen and mesenteric lymph node in mice

The mice were sacrificed on day 31. The spleens and mesenteric lymph node (MLN) were removed under sterile conditions and ground in RPMI-1640 medium containing 10% FBS. The splenocytes and MLN lymphocytes were obtained and collected, respectively. An appropriate volume of RCLB was added into the splenocytes. After treatment with RCLB, the supernatant was discarded after centrifugation (2000 rpm, 4°C, 5 min). The lymphocytes were collected before being resuspended in 10% FBS-RPMI 1640 medium at a concentration of 1×10^6 cell·ml^{-1}.

Flow cytometry analysis

For Treg cells frequency analysis, we mixed the cells with 5 μl anti-mouse CD4 FITC and 5 μl anti-mouse CD25 APC and incubated the mixture at 4°C in darkness for 30 min. After the first incubation, the cells were stained with 3 μl anti-mouse Foxp3 PE, or 2.5 μl PE-Rat IgG2a as control, incubated at 4°C for 30 min in darkness. For the Th17 cells analysis, the cells were stimulated with 50 ng/ml PMA/Ionomycin plus 500 ng/ml BFA/Monensin, then stained with 5 μl anti-mouse CD4 FITC before being fixed and permeabilised. After permeabilisation, the cells were incubated with 5μl anti-Mouse/Rat–IL-17A PE, or PE- Rat IgG2a as control, at 4°C for 20 min in the dark. After staining, we analysed the percentage of cells with the different labels on a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson).

Detection of transforming growth factor-β and interleukin -17A from the spleen, mesenteric lymph node and serum

Serum was collected on day 31 and used immediately to determine cytokine levels. On the other hand, the lymphocytes from spleen and MLN were diluted to a concentration of 1×10^6 cell·mL^{-1}. For the intracellular cytokine analysis, the cells were stimulated with 50 ng/ml PMA plus 500 ng/ml ionomycin for 4–5 hours in the presence of 1μg/ml BFA. The supernatant was collected. The levels of TGF-β and IL-17A were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.

Histopathological analysis of arthritis

The paws from each mouse were collected under sterile conditions and cut into sections. The tissue slices were then stained with haematoxylin and eosin (HE). The histological analysis was assessed microscopically by 2 independent observers. The severity of

arthritis was evaluated and scored individually on the following scale: 0 = normal synovium, 1 = synovial membrane hypertrophy and cell infiltrates, 2 = pannus and cartilage erosion, 3 = major erosion of cartilage and subchondralbone, and 4 = loss of joint integrity and ankylosis.

Analysis of regulatory T cell and T-helper 17 cell in synovial tissue

The presence of Treg and Th17 cells in the synovial tissue was measured by direct immune-fluorescence (DIF). Joint tissue sections were fixed in formaldehyde. After hydration and dewaxing, the tissue slices were incubated with 3% BSA to block the non-specific binding sites. Then, all of the tissue slices were stained to mark Treg cells with FITC-labelled anti- CD4 (1:100) and PE-labelled anti- Foxp3 (1:100), while the Th17 cells were stained with FITC-labelled anti-CD4 (1:100) and PE-labelled anti-IL-17A (1:100). After an overnight incubation at 4°C, the cell nuclei were stained with DAPI. An anti-fluorescence quenching agent was used as a mounting medium. We observed the results of the double immunofluorescence staining with Leica inverted fluorescence microscope and saved images after observation. Cell counting was performed under microscopes (10×), and only the morphologically intact cells were counted as target cells. 6 slices were made in each group, and the number of targey cells among 100 cells was counted in each slice by microscope.

Statistical analysis

The data analyses were performed using SPSS software v. 13.0. The results are expressed as the mean ± SD. The statistical differences between the percentage of Treg and Th17 cells were analysed using analysis of variance (ANOVA) with LSD adjustment, while the differences between the histopathological scores were analysed by ANOVA with Tamhane adjustment. p-values <0.05 were considered significant.

Results

Purification and identification of zaocys type II collagen

The molecular weight of ZCII was about 110KD~140KD determined by SDS-PAGE analysis, and the UV absorption peak of ZCII centered around 230 nm detected by ultraviolet spectrophotometer. The data of both results were similar to the standard CCII (Fig. 1A-B).The PMF of ZCII obtained by MS analysis showed that at least 4 peptides matched with other species. The protein score was greater than 95%, demonstrating that the collagen was CII (Fig. 1C).

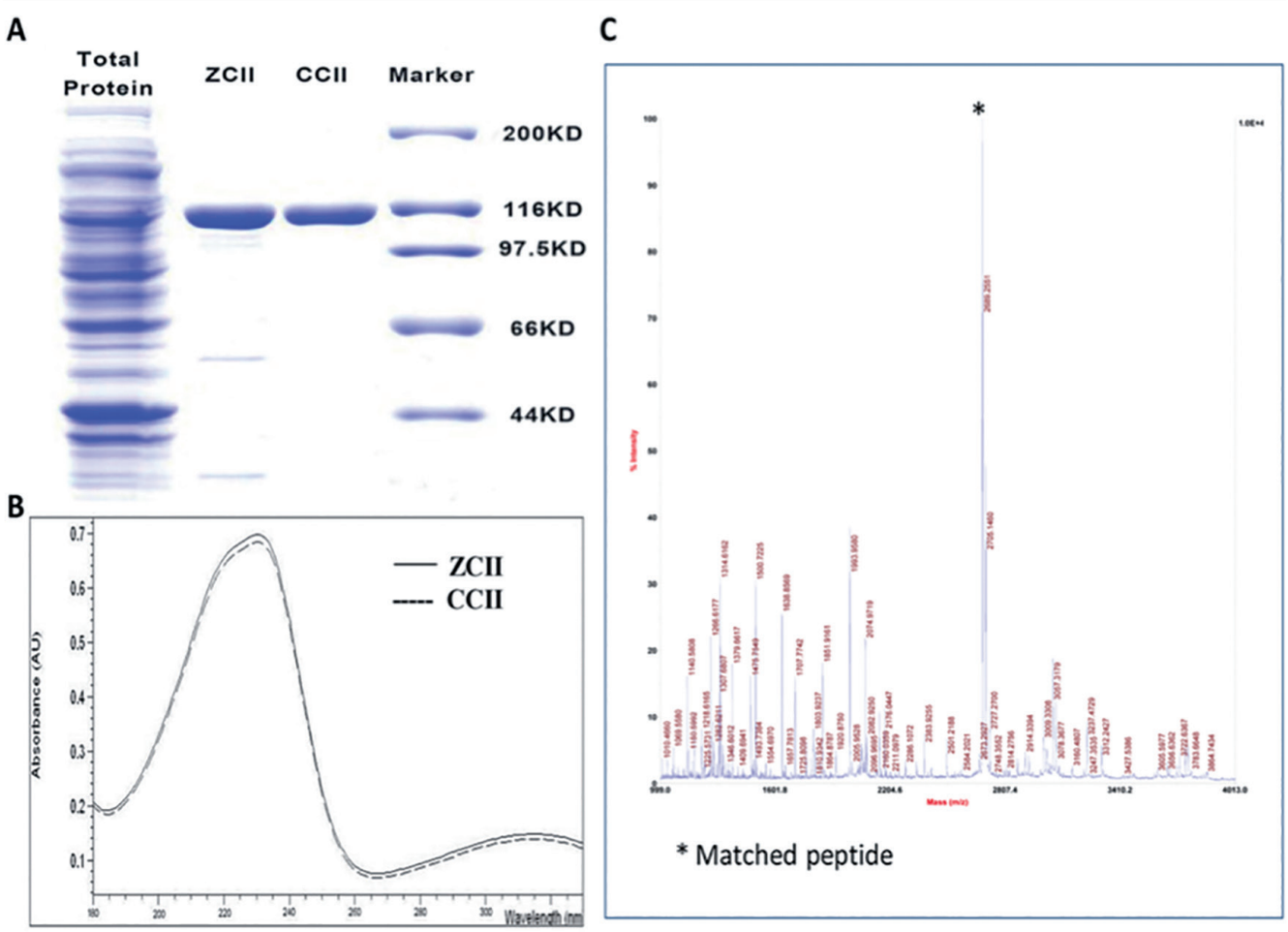

Fig. 1. Identification of zaocys type II collagen. (**A**) The molecular weight of ZCII was detected by SDS-PAGE analysis with standard CC II. (**B**) The ultraviolet absorption of ZCII was detected with an ultraviolet spectrophotometer, with CCII. (**C**) The PMF of ZCII was analysed by mass spectrometry.

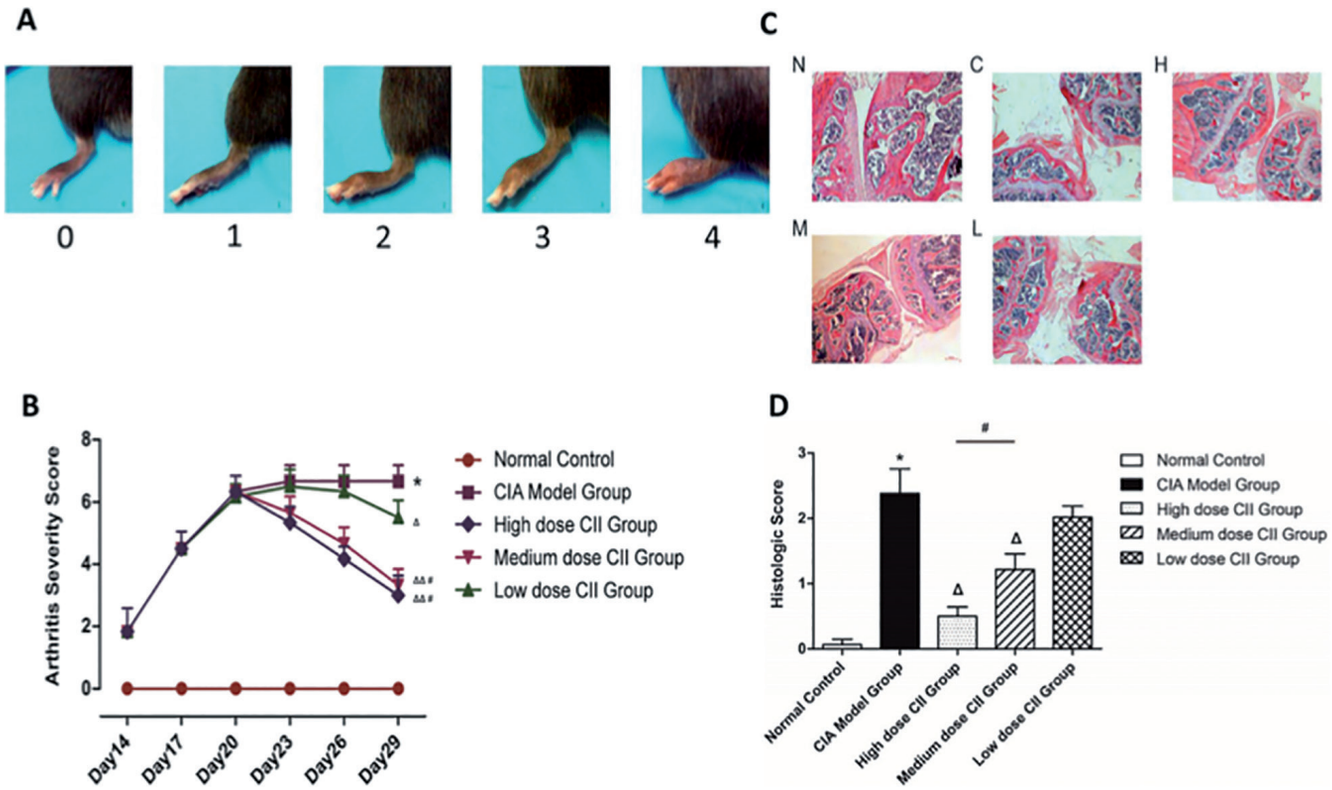

Fig. 2. The therapeutic effect of zaocys type II collagen on mice with collagen-induced arthritis. (**A**) The standard of arthritis severity score. 0 = no swelling and focal redness, 1 = swelling of finger joints, 2 = mild swelling of ankle or wrist joints, 3 = severe inflammation of the entire paw, and 4 = deformity or ankylosis. (**B**) The arthritis severity score of each mouse in the normal control group, CIA model group, high, medium and low dose CII groups was recorded every three days starting on day 14 after arthritis induction. (*p<0.01; Δ: p<0.05; ΔΔ: p<0.01; # intergroup comparison p<0.05). (**C**) Histological sections of synovium were stained with haematoxylin and eosin. Original magnification ×40. (**D**) The histological score for the synovium of arthritis severity. Normal mice: zero. The severity of arthritis was evaluated and scored individually on the following scale: 0 = normal synovium, 1 = synovial membrane hypertrophy and cell infiltrates, 2 = pannus and cartilage erosion, 3 = major erosion of cartilage and subchondral bone, and 4 = loss of joint integrity and ankylosis. (*both p<0.01; Δ: all p<0.01 *versus* CIA group; all p<0.01 among treatment groups).

The therapeutic effect of zaocys type II collagen on mice with collagen-induced arthritis

The mice were monitored for signs of arthritis by 2 independent observers on day 14, 17, 20, 23, 26 and 29 after the injection with CCII. ZCII was orally administered to the mice on day 21 after immunisation. The results showed a significant increase in arthritis score in the CIA group compared to the normal control group ($^{*}p$<0.01). Treatment with ZCII markedly reduced the arthritis score of the mice suggesting the alleviation of arthritis ($^{\Delta}p$<0.05; $^{\Delta\Delta}p$<0.01). The mice from the high and medium dose groups had lower arthritis scores than the mice from the low dose ZCII treatment group ($^{\#}$intergroup comparison p<0.05). Although the high dose of ZCII seemed to be more effective in lowering the score, no significant statistical difference was found between the results obtained with the high and medium dose ZCII treatments (p>0.05) (Fig. 2A-B). These results suggested that ZCII could remarkably suppress the severity of arthritis.

The therapeutic effect of ZCII on CIA mice was further verified by histological examination. In the CIA group, the histological score increased to 2.38±0.38 ($^{*}p$<0.01). The low dose of ZCII treatment lowered the score to 2.01±0.93. However, the difference was not significant between the low dose group and the CIA model group (p>0.05). The scores were significantly lowered to 1.22±0.24 in medium dose group, and 0.50±0.14 in high dose group ($^{\Delta}$all p<0.01 *vs*. CIA group; $^{\#}$all p<0.01 among treatment groups). Compared with the normal group, the mice in the model group had obvious clinical signs. In contrast, the joint tissues of ZCII-treated mice exhibited a remarkable reduction in inflammatory cell infiltration and pannus formation. In addition, ZCII also significantly ameliorated other clinical symptoms, including cartilage destruction and bone erosion (Fig. 2C-D).

Treatment with zaocys type II collagen increased the regulatory T cell frequency but reduced the T-helper 17 cell frequency

The Treg and Th17 cells percentage splenocytes and MLN lymphocytes were analysed by flow cytometry. The normal Treg percentage was 7.42±1.07% in splenocytes and 6.41±1.01% in MLNLs. In the CIA model group, the proportion of Tregs increased significantly to 15.58±1.25% in splenocytes and 13.70±1.85% in MLNLs (*both p<0.01). Treatment with ZCII caused a remarkable increase in the Treg cells in splenocytes, increasing the proportion to 33.95±1.79% in the high dose group, 26.49±1.44% in the medium dose group and 20.73±2.17% in the

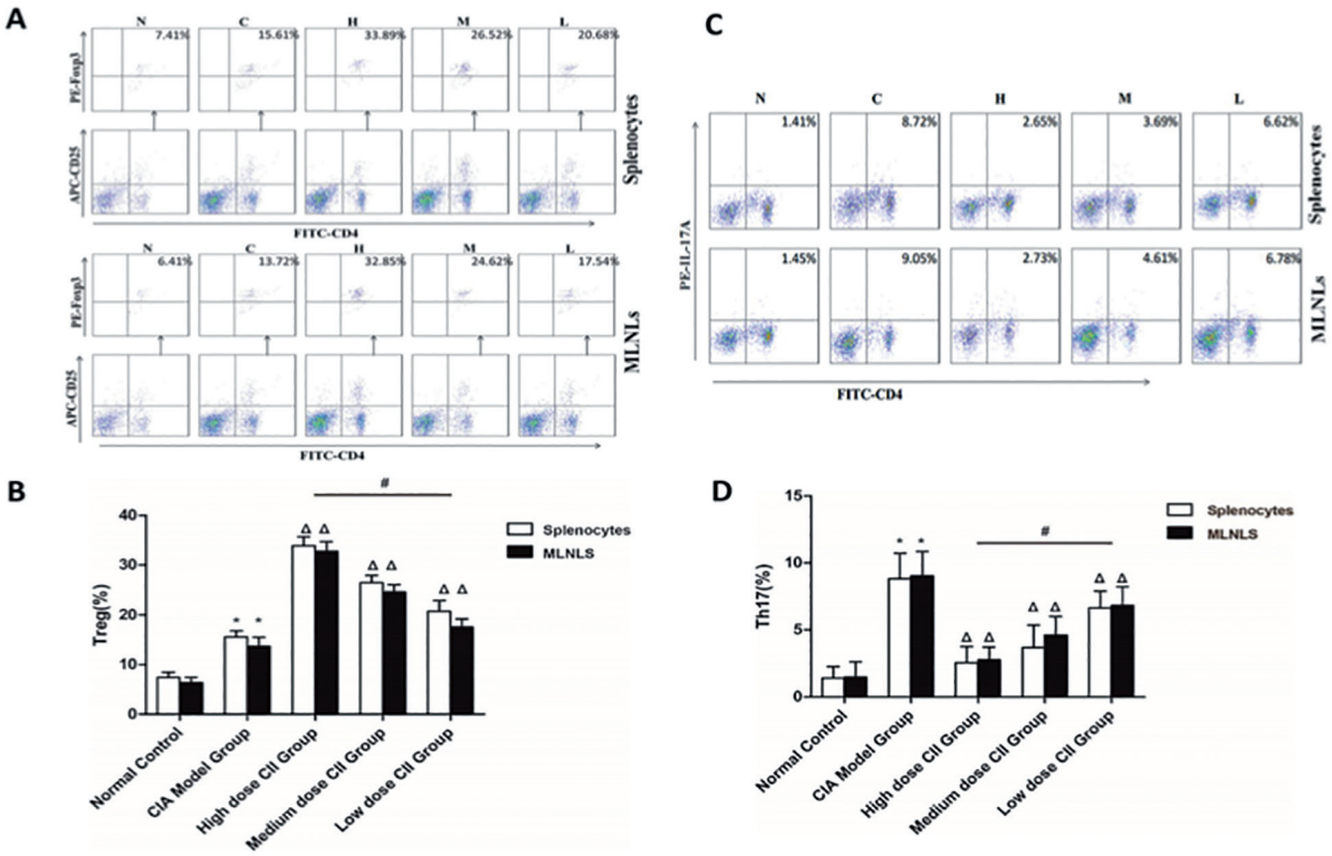

Fig. 3. The flow cytometry analysis of regulatory T cells and T-helper 17 cells in zaocys type II collagen-treated mice. (**A**) The Flow cytometry analysis of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells. (**B**) ZCII groups dose-dependently increased the percentage of Treg cell in the lymph tissues of CIA mice. (**C**) The flow cytometry analysis of $CD4^+IL\text{-}17A^+Th17$ cells. (**D**) ZCII groups dose-dependently inhibited the proportion of Th17 in the lymph tissues of CIA mice. (*both $p<0.01$; Δ: all $p<0.05$ *vs*. CIA group; #all $p<0.05$ among treatment groups).

low dose group (Δall $p<0.05$ *vs*. CIA group; #all $p<0.05$ among treatment groups). The treatment with ZCII also caused significant elevation in Treg percentage in the MLNLs, increasing the proportion to 32.83±1.94% in the high dose group, 24.59±1.51% in the medium dose group and 17.58±1.60% in the low dose group (Δall $p<0.05$ *vs*. CIA group; #all $p<0.05$ among treatment groups) (Fig. 3A-B). In brief, the percentage of Treg cells among the $CD4^+$T cells in splenocytes and the MLN lymphocytes was increased in the model group compared with the normal group. Treatment with ZCII significantly increased the frequency of Treg cells.

The normal Th17 proportion was 1.41±0.85% in splenocytes and 1.47±1.16% in MLNLs. In the CIA model group, the proportion of Th17 cells markedly increased to 8.82±1.91% in splenocytes and 9.03±1.83% in MLNLs (*both $p<0.01$). Treatment with ZCII drastically lowered the Th17 percentage in splenocytes to 2.55±1.21% in the high dose group, 3.71±1.65% in the medium dose group and 6.65±1.24% in the low dose group (Δall $p<0.05$ *vs*. CIA group; #all $p<0.05$ among treatment groups). A similar decrease was observed in MLNLs, with the Th17 percentage decreased to 2.78±0.94% in the high dose group, 4.63±1.36% in the medium dose group and 6.85±1.37% in the low dose group (Δall $p<0.05$ *vs*. CIA group; #all $p<0.05$ among treatment groups) (Fig. 3C-D). In conclusion, the frequency of Th17 cells in the splenocytes and MLN lymphocytes of CIA mice was also increased compared with the normal group, but the ZCII-treated mice showed a decrease in the number of Th17 cells.

Regulatory T cell and T-helper 17 cell populations in the synovial tissue

We determined the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells and $CD4^+IL\text{-}17A^+$ Th17 cells in the synovial tissues of mice treated with ZCII.

The number Th17cells in the synovial tissue of CIA mice was higher than the normal group. (*$p<0.01$ *vs*. normal control) Treatment with ZCII increased the Treg cells in synovial tissue to 33.07±5.14% in the high dose group, 29.05±3.24% in the medium dose group and 24.31±4.52% in the low dose group (Δ$p<0.01$ *vs*. CIA group; #$p<0.05$ inter-group comparison). Decrease of Th17 cells was observed in synovial tissue, with Th17 percentage decreased to 5.63±1.76% in the high dose group, 7.54±3.68% in the medium dose group and 8.96±2.98% in the low dose group (Δ$p<0.01$ *vs*. CIA group; #$p<0.05$ inter-group comparison). These results demonstrated that ZCII increased the number of Treg cells and reciprocally decreased the number of Th17 cells compared with the model group (Treg cell: 23.12±4.93%, Th17 cell: 9.74±2.23%) (Fig. 4A, B, C).

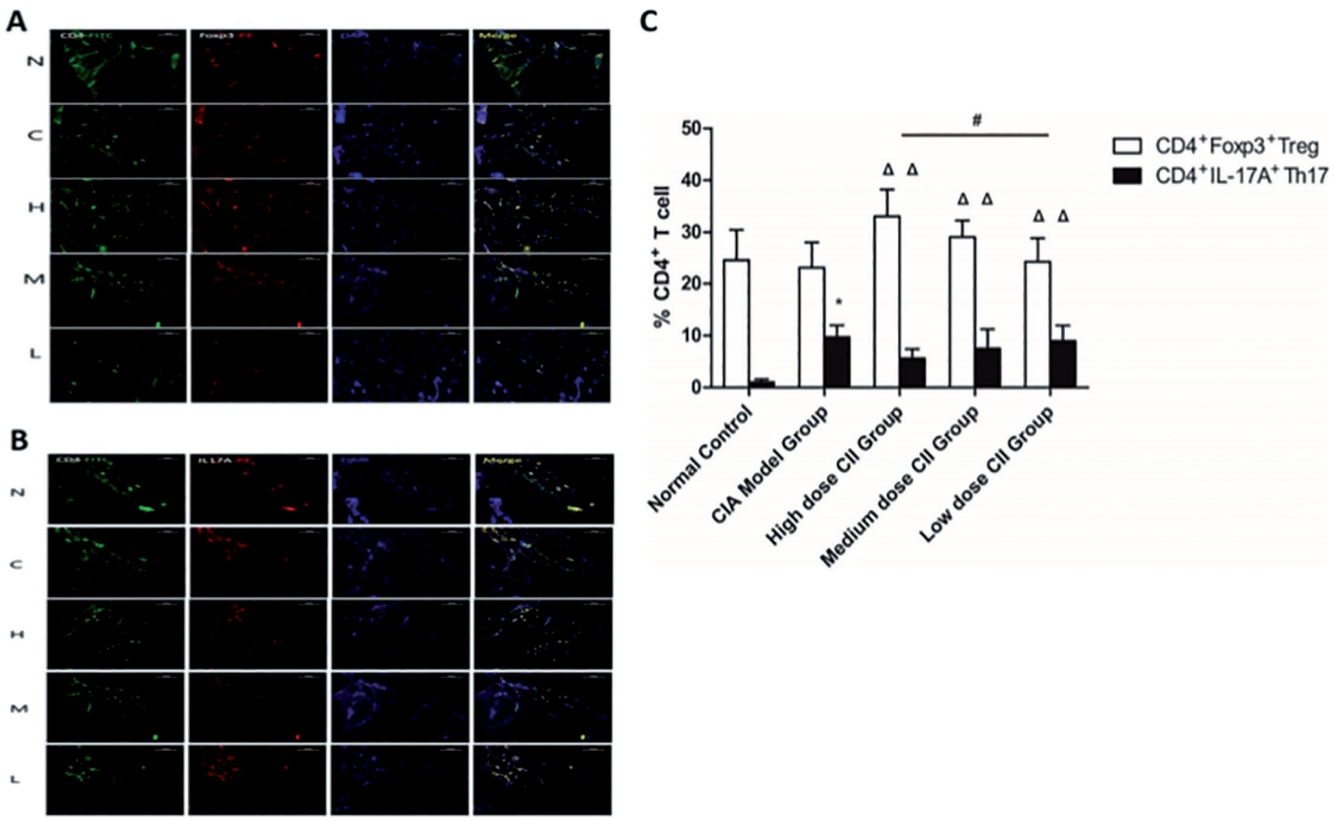

Fig. 4. The direct immune-fluorescence results of regulatory T cells and T-helper 17 cells in the synovial tissue. (**A**) All of the tissue slices were stained for Treg cell analysis with FITC-labelled anti-CD4 (1:100) and PE-labelled anti- Foxp3 (1:100), the cell nuclei were stained with DAPI. Cell counting was performed under microscopes (10×), and only the morphologically intact cells were counted as positive cells. The number of positive cells in 100 cells was counted in each slice by microscope. (**B**) Th17 cells were stained with FITC-labelled anti-CD4 (1:100) and PE-labelled anti-IL-17A (1:100), the cell nuclei were stained with DAPI, cell counting was the same as above. (**C**) The numbers of Th17 cells in the synovial tissue of CIA mice were higher than those in the normal group. Treatment with ZCII caused a remarkable increase in the Treg cells when drastically lowered the Th17 percentage in synovial tissue (**p*<0.01 *vs*. normal control; Δ: *p*<0.01 *vs*. CIA group; #*p*<0.05 inter-group comparison)

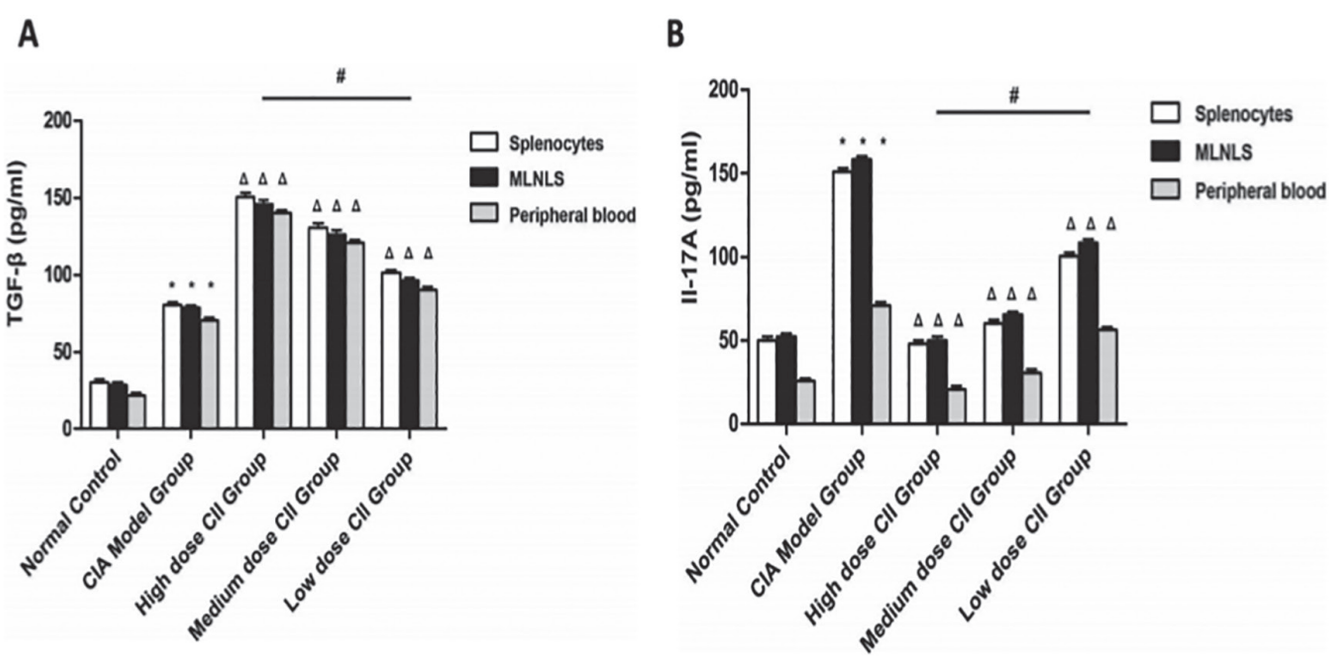

Fig. 5. The effect of zaocys type II collagen on transforming growth factor-β and interleukin -17A in mice with collagen-induced arthritis. (**A**) ZCII groups dose-dependently enhanced TGF-β secretion in mice with CIA. (**B**) ZCII groups dose-dependently inhibited Il-17A secretion in the lymph tissues of CIA mice. (*all *p*<0.01 *vs*. normal control; Δ: all *p*<0.01 *vs*. CIA group; #all *p*<0.01 among treatment groups).

The effect of zaocys type II collagen on transforming growth factor-β and interleukin-17A in mice with collagen-induced arthritis

The normal TGF-β secretion level was 30.50±1.88 pg/ml in splenocytes, 28.46±1.87 pg/ml in MLNLs and 21.77±1.61 pg/ml in the peripheral blood. In the CIA model group, the level of TGF-β significantly increased to 80.62±1.78 pg/ml in splenocytes, 78.72±1.73 pg/ml in MLNLs and markedly 70.58±1.76 pg/ml in peripheral blood (*all $p<0.01$ *vs*. normal control). Treatment with ZCII significantly increased the TGF-β secretion in splenocytes to 150.36±2.89 pg/ml in the high dose group, 130.67±2.89 pg/ml in the medium dose group and 101.21±1.86 in the low dose group ($^{\Delta}$all $p<0.01$ versus CIA group, $^{\#}$all $p<0.01$ among treatment groups). A similar elevation was noticed in MLNLs and the peripheral blood. In MLNLs, the concentration of TGF-β was 145.86±2.74 pg/ml in the high dose group, 126.03±3.15 pg/ml in the medium dose group and 96.35±1.93 pg/ml in the low dose group ($^{\Delta}$all $p<0.01$ *vs*. CIA group; $^{\#}$all $p<0.01$ among treatment groups). In the peripheral blood, the concentration of TGF-β was 140.17±1.78 pg/ml in the high dose group, 120.90±1.76 pg/ml in the medium dose group and 90.25±2.08 pg/ml in the low dose group ($^{\Delta}$all $p<0.01$ *vs*. CIA group; $^{\#}$all $p<0.01$ among treatment groups). In brief, compared to the normal group, CIA mice had a higher level of TGF-β in the serum and the lymphocytes supernatant from the spleens and MLN ($p<0.05$). In addition, ZCII groups also had a higher level of TGF-β than the model group ($p<0.05$) (Fig. 5A). The normal level of IL-17A was 50.33±2.23 pg/ml in splenocytes, 52.35±2.15 pg/ml in MLNLs and 25.75±1.30 pg/ml in the peripheral blood. In the CIA model group, the IL-17A level markedly increased to 151.05±1.98 pg/ml in splenocytes, 158.44±1.70 pg/ml in MLNLs and 70.08±1.98 pg/ml in the peripheral blood (*all $p<0.01$ *vs*. normal control). Treatment with ZCII resulted in remarkable lower levels of IL-17A in splenocytes: 48.33±1.74 pg/ml in the high dose group, 60.39±1.99 pg/ml in the medium dose group and 100.39±2.24 pg/ml in the low dose group ($^{\Delta}$all $p<0.01$ *vs*. CIA group; $^{\#}$all $p<0.01$ among treatment groups). A similar suppression was noticed in MLNLs and the peripheral blood. The IL-17A concentrationin MLNLs was 50.23±2.17 pg/ml in the high dose group, 65.51±1.61 pg/ml in the medium dose group and 108.54±1.88 pg/ml in the low dose group ($^{\Delta}$all $p<0.01$ *vs*. CIA group; $^{\#}$all $p<0.01$ among treatment groups). In the peripheral blood, the concentration of IL-17A was 20.73±2.03 pg/ml in the high dose group, 30.72±2.10 pg/ml in the medium dose group and 56.52±1.54 pg/ml in the low dose group. Meanwhile, the CIA mice had a higher level of IL-17A compared with the normal group. In contrast, ZCII treatment significantly decreased the production of IL-17A compared with the model group ($p<0.05$) (Fig. 5B).

Discussion

CIA is now widely accepted as a valid animal model of human RA. In this study, we found that ZCII significantly reduced the severity of arthritis in CIA mice. And histopathological studies showed that oral administration of ZCII ameliorated synovial hyperplasia, monocyte infiltration, the formation of pannus and the erosion of cartilage and bone tissue. The findings above suggested that ZCII might be a promising candidate for the treatment of RA.

Oral tolerance is known that the primary mechanism mediating oral tolerance is the suppression of immune reactions by Treg cells (32). Treg cells can produce TGF-β, a cytokine with known regulatory functions (12). Treg cells can also function as important regulatory factors and express latency-associated peptide (LAP) on their surface. The expression of the LAP-TGF-β complex on the surface of Treg cells plays a crucial role in controlling RA (33). The major function of Treg cell is regulating the function of other T lymphocytes, to avoid excessive immune activation and then control autoimmune disease or auto-inflammatory reactions. Recently, the induction of oral tolerance has been intensively investigated and utilised in studies on autoimmune diseases in experimental animal models and human (34).

In this study, we purified CII from zaocys and proved that this collagen is homologous to CCII. We also observed the effect of ZCII on Treg cells in the MLN and spleen of CIA mice. The results demonstrated that oral administration of ZCII increases the proportion of Treg cells by promoting the differentiation of $CD4^{+}$T cells into Treg cells. Additional experiments also confirmed that oral administration of ZCII could enhance the expression of TGF-β in CIA mice, thereby inhibiting the function of T cells and antigen presenting cells (APC) indirectly (17). TGF-β induces the production of Treg cells, upregulates the expression of inhibitory immune cell surface molecules and suppresses the expression of genes that activate T cells, thus exerting its immunoregulatory function (35).

TGF-β is a homodimeric protein with pleiotropic actions in immune responses, inflammation and repair (36). Studies show that TGF-β can inhibit the formation and activity of osteoclast, stimulate the activity of osteoblast (37), facilitate fibroblast to produce collagen and fibronectin, and stimulate angiogenesis (38), which might be related to the remodelling and repair of bone and tissue of RA. As mentioned previously, TGF-β is associated with the development of Th17 cells in mice (40). Besides, TGF-β was considered to suppress the development of Th1 and Th2 cells by inhibiting lineage-specific transcription factors, including T-bet and GATA-3, which indicated that TGF-β affects Th17 cell differentiation as an indirect effector (14, 41). In this study, we analysed the proportion of Th17 cells after various treatments and found an increased number of Th17 cells in the MLN and spleen of CIA mice. The other effects of TGF-β on RA need further validation.

We further observed the effect of ZCII on Th17 cells in the MLN and spleen of CIA mice. The results of this experiment indicated that oral administration of ZCII reduced the proportion of Th17 cells by inhibiting the differentiation of

$CD4^+$T cells into Th17 cells. Th17 cells can produce IL-17A, which is a pro-inflammatory cytokine. And IL-17A induces the synthesis of TNF-α, IL-1β, IL-6, IL-8 and GM-CSF. It also promotes synovial inflammation and cartilage damage to the joints. Besides, IL-17A is associated with the destruction of the extracellular matrix and juxta articular bone resorption in patients with RA, through the induction of synthesis of RANKL and MMP synthesis (42, 43). In this work, we demonstrated that ZCII suppressed the generation of IL-17A in the serum and MLN and spleen. A deficiency of IL-17A would inhibit the induction and development of CIA in mice (44). Consequently, joint inflammation, cartilage destruction, and bone erosion are supposed to be ameliorated through neutralising IL-17A in animals with CIA.

It is known that macrophages and synovial fibroblasts are effector cells in joint destruction, and auto-reactive $CD4^+$ T cells are involved in the activation of these effector cells (45). Studies investigating the location and proportion of Treg/Th17 cells in joints are quite rare nowadays. This work has observed the expression of Treg and Th17 cells in the joints of CIA mice by DIF, finding that Treg and Th17 cells are primarily present in the synovial lining layer or around the blood vessels. The proportion of Th17 cells in CIA mice was significantly higher than the normal group, but there was no difference in Treg cell count between two groups. This result revealed that the differentiation of naive T cells into Treg/Th17 cells was not balanced in the model group. This study also showed that oral administration of ZCII could increase the proportion of Treg cells and reduce the proportion of Th17 cells present in the joints.

Conclusion

In conclusion, the oral administration of ZCII ameliorated CIA by increasing the proportion of Treg cells and suppressing the production of Th17 cells to regulate the immune balance in the spleen, MLN and synovial tissue. Therefore, ZCII may have great potential as a promising treatment for RA patients.

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