# Effectiveness of methotrexate in combination therapy in a rat collagen-induced arthritis model

**Chenchen Zhuang[2], Juan Zhou[1], Hailu Mo[1], Dong Lin[1], Xiaohong Luo[1] and Hanyou Mo[1]***

[1]Department of Clinical Immunology and Rheumatology, Guilin Medical University Affiliated Hospital, Guilin, China
[2]Lanzhou University Second Hospital, Lanzhou, China

**Abstract**: This study was to investigate the effect of methotrexate in combination therapy by the characteristic cytokine in Th17 cells and the frequency of Tregs, which involved in the induction and pathological progress of rheumatoid arthritis (RA). The collagen-induced arthritis rats were treated with methotrexate + prednisone, methotrexate + disease-modifying rheumatic drugs (DMARDs) and methotrexate + TNFi, respectively. The following parameters were observed to evaluate three treatments: the frequency and function of Th17 cells and Tregs, the scores of X-rays, H&E staining and immunohistochemistry. For rats starting methotrexate + prednisone (low doses), the frequency and suppressive function of Th17 cells decreased while the frequency of Tregs increased, which were the same in methotrexate + TNFi. Immunohistochemical in the pathological sections of ankle joint showed the same results. The effect of methotrexate + DMARDs treatment was slightly inferior to the other combination therapies. In summary, rats treated with methotrexate + prednisone can achieve high level of Tregs and low level of Th17 cells and IL-17. Low doses of glucocorticoid suggesting a critical role in the pathogenesis of rheumatoid arthritis may have the similar effect as DMARDs.

**Keywords**: Prednisone, methotrexate, IL-17, Th17, Tregs

## INTRODUCTION

RA is associated with local production of pro-inflammatory cytokines, such as IL-1 and IL-17, resulting in joint synovitis (Kirim *et al*., 2010; Benedetti and Miossec, 2014). MTX is the anchor drug in the treatment of RA and recommended as the first-line therapy, which is regarded as an essential component of combination therapies (Nam *et al*., 2010; Smolen *et al*., 2010; Elisabeth *et al*., 2011; Wu *et al*., 2016). Various high quality clinical studies have proved that MTX should be added to DMARDs or TNFi (Fleischmann *et al*., 2014; Machado *et al*., 2014; Kou *et al*., 2015). ETN, a soluble TNFα receptor immunoglobulin fusion protein, has been recognized as an important key in the pathogenesis of RA and combination with MTX (Weinblatt *et al*., 1999; Emery *et al*., 2014). So this is the reason we choose ETN to represent TNFi drugs in this experiment.

The study compared the effect of the combination therapies with Pred, DMARDs and TNFi in MTX, and a critical question is whether the Pred had DMARDs effects. Some studies revealed that radiological progression was suppressed in early RA patients administered with low doses of glucocorticoid. The combination of Pred or DMARDs in MTX increased the effect of DMARDs and the DMARDs usage, which reduced its side effects (Graudal and Jürgens, 2010; Verschueren *et al*., 2015; Wasko *et al*., 2016). The combination therapy may be feasible for RA, especially with vasculitis or interstitial lung disease (Gaujoux Viala and Gossec, 2014). Therefore, related studies through animal experiments are essential. Particularly, Th17 cells and Tregs are critical for monitoring disease severity in autoimmunity, which is closely associated with RA progression. Foxp3 is essential in Tregs development, survival, and function, whereas IL-17 (the prototypic Th17 cell pro-inflammatory cytokine) is involved in the induction and pathological progress of RA (Smolen *et al*., 2010; O'dell *et al*., 2013; Furst and Emery, 2014; Noack and Miossec, 2014). IL-17 produced by Th17 cells had high abilities to stimulate acute and chronic inflammation, which is regarded as ideal candidate for important player in the development of RA. Thus, reduced Foxp3 or enhanced IL-17 activity could be mediated to accelerate RA progression. However, few studies have been conducted to evaluate the variation of Th17 and Tregs in MTX in combination therapy. The aim of this study was to examine the correlation between the amount of Th17 and Tregs and the combination therapies, and the effect of the MTX + Pred treatment.

## MATERIALS AND METHODS

### *Experimental animals*

61 four-weeks-old male Sprague Dawley rats, weighting approximately 100g, were obtained from the Guilin Medical College Experimental Animal Center.

### *Main reagents*

Collagen type II: Chondrex Co, 5ml/bottle; CFA: Sigma Co, 10ml/bottle; MTX, Pred, HCQ, SSZ, ETN (Enbrel; Guilin pharmaceutical Co. LTD); Leukocyte Activation Cocktail, with BD GolgiPlug: BD Pharmingen Co; Lysing Buffer: BD Pharm Lyse Co; Anti-Rat IL-17A PE, Anti-Rat Foxp3 PE, Anti-Rat CD4 APC, Rat IgG2a Isotype

**Corresponding author:* e-mail: chairperson.scps@stmu.edu.pk

Control PE, Foxp3/Transcription Factor Staining Buffer Set: eBioscience Co; Anti-IL-17 antibody, Anti-Foxp3 antibody: Abcom. Co; the biotin-labeled secondary antibody (sheep anti-rabbit IgG): Pathology laboratory of Guilin Medical University Affiliated Hospital; ELISA kit for IL-17A was purchased from R&D Systems, Inc.

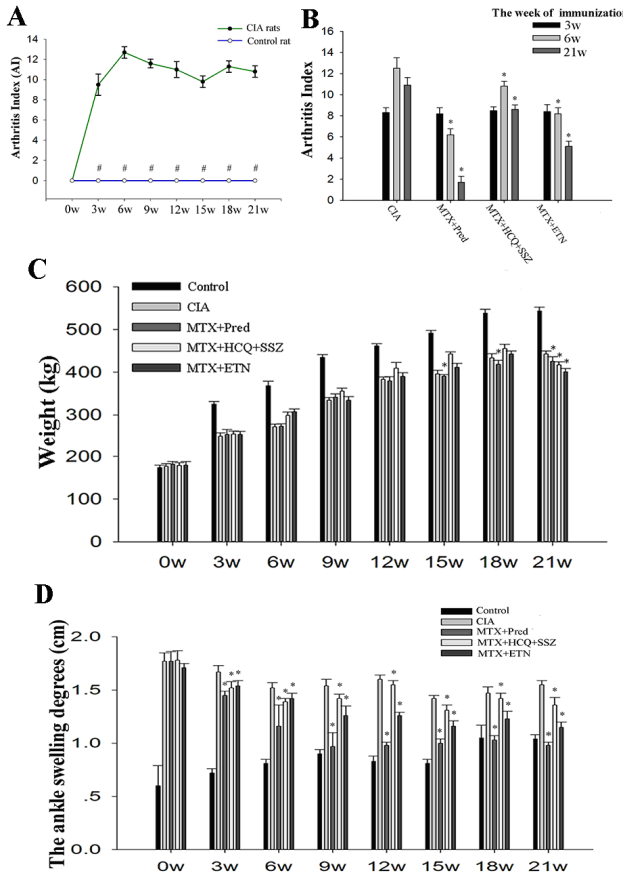


**Fig. 1**: Changes in the CIA rats and the three treatment groups. (A) 'AI' of the CIA rats without any drugs, (B) 'AI' of the CIA rats in each treatment group, (C) the weight of the CIA rats in each treatment group, (D) the ankle swelling of the CIA rats in each treatment group. Note: $^{*}P<0.05$, *vs.* the CIA rats at the same time.

## *Methods*

*Preparation of the CIA model*

Prior to the experiment, type Ⅱ collagen (including acetic acid with a concentration of 2mg/ml) was added slowly to the same volume of CFA, prepared in sufficient emulsification on ice and the final concentration of the mixture was 1mg/ml. The rats administered by pentobarbital sodium anesthesia, were immunized intradermally twice at the base of the tail and the back at intervals of 2 weeks, while the control was intragastrically administered with saline (Ferry *et al*., 2013). Analysis was performed once a week after primary immunity.

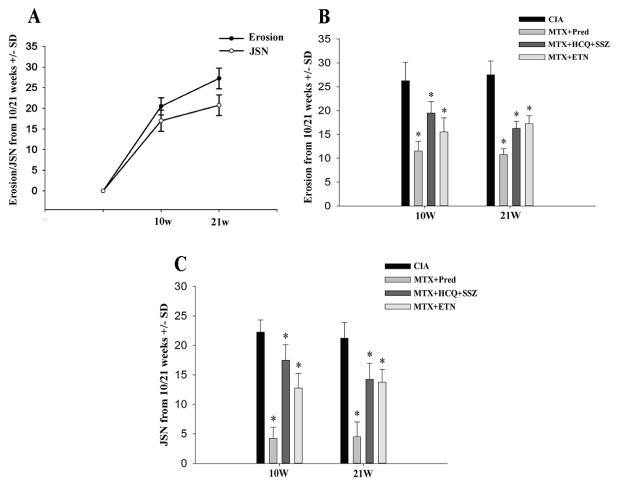


**Fig. 2**: Effects of combination therapies in each group evaluated by radiography. (B) semi-quantification of erosion/JSN in CIA model, (D) semi-quantification of erosion in each treatment group, (F) semi-quantification of JSN in each treatment group. Note: $^{*}P<0.01$, *vs.* the CIA group at the same time.

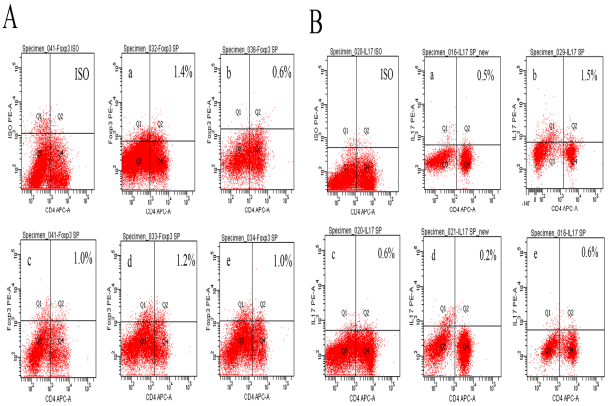


**Fig. 3**: (A) Flow cytometry detection diagram of Tregs in the control group, the CIA group, the MTX + Pred group, the MTX + HCQ + SSZ group, the MTX + ETN group (a, b, c, d and e, respectively), (B) Flow cytometry detection diagram of Th17 in the control group, the CIA group, the MTX + Pred group, the MTX + HCQ + SSZ group, the MTX + ETN group (a, b, c, d and e, respectively)

**Table 1**: IL-17 serum in rats at week 10 and 21 (mean ± SD, pg/ml)

| Groups | 10th week | 21th week |
|---|---|---|
| Control | 11.3±1.6 | 12.1±1.8 |
| CIA | 89.5±4.9 | 82.7±4.4 |
| MTX+Pred | 47.8±2.4$^{a,b}$ | 35.5±5.5$^{a,b}$ |
| MTX+HCQ+SSZ | 61.2±3.3$^{a}$ | 57.7±5.7$^{a}$ |
| MTX+ETN | 44.7±4.5$^{a,b}$ | 34.6±5.2$^{a,b}$ |

Note: $^{a}P<0.01$, *vs.* the CIA group; $^{b}P<0.01$, *vs.* the MTX+HCQ+SSZ group

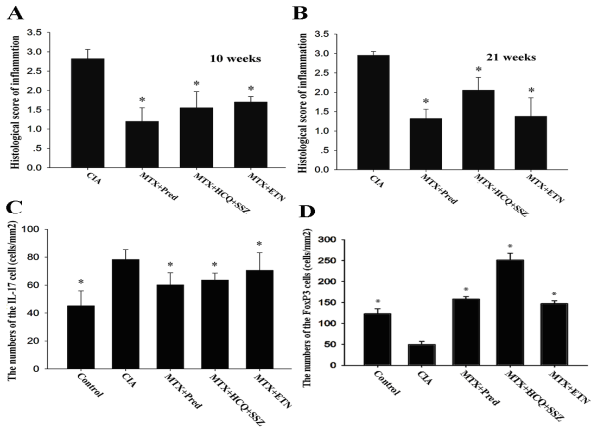


**Fig. 4**: Effects of combination therapies in each group by histological scores and immunohistochemial staining. (A) Histological scores of inflammation at 10 weeks, (B) Histological scores of inflammation at 21 weeks, (C) Semi-quantification of IL-17-positive cells, (D) Semi-quantification of Foxp3-positive cells. Note: $^{*}P<0.05$, *vs.* the CIA group. Data are expressed as mean ± SD.

***Treatments***

There were five groups of CIA rats: Group A-Control group without CIA treatment or any drugs, Group, B-CIA treatment without any drugs, Group C-CIA treatment with MTX (2.7mg/kg/week) + Pred (4mg/kg/day), Group D-CIA treatment with MTX (2.7mg/kg/week) + HCQ (40 mg/kg/day) + SSZ (160mg/kg/day), Group E-CIA treatment with MTX (2.7mg/kg/week) + ETN (0.3mg/kg/day). All these rats had an 'AI' score of more than 6 points, and feeding and drug administration were carried out separately.

Primary immunization was set as day 0. Drug administration began 14 days after primary immunization, and all rats were sacrificed to the last 21 weeks. MTX (Inoue and Yuasa, 2014), Pred (Xu *et al*., 2008), HCQ (Furst, 1996), and SSZ (Chungi *et al*., 1989) were administered by gavage, and ETN (Zhou, 2005) was subcutaneously injected twice a week. Rats in the control group and CIA group were given equal amounts of physiological saline as the other groups.

***Radiological examination and assessment***

At week 10 and 21 after primary immunity, the radiological examination was performed on the left posterior ankle. X-rays of rat ankle joints were evaluated according to the SHS. Semi-quantitative scoring was calculated by the coordinating center from Guilin Medical University Affiliated Hospital. The joint area was scored for JSN and erosions (Smolen and Steiner, 2003; Ma *et al*., 2015).

***Flow cytometry***

Rats were sacrificed and extracted of spleen lymphocytes. The spleen cells was harvested with $10^6$ cells/ml. Intracellular staining for FoxP3 and IL-17 were performed according to the appropriate eBioscience protocol (Tian *et al*., 2013). All tubes were added Leukocyte Activation Cocktail with BD Golgi Plug for 6h. Then each tube was added FACS buffer (including 0.05% Azide in 1xPBS and 0.5% BSA), and centrifuged at 1500 r/min for 5 minutes. For cell surface staining, the tube was added CD4-APC and plused vortex to each tube at 4°C for 30 minutes in the dark. Each tube was added Foxp3 at 4°Cfor 40 minutes in the dark to fix and rupture of membranes. Without washing, each tube was added Permeabilization Buffer with 1500 r/min centrifugation for 5 minutes, then resuspended in 100µl of 1X Permeabilization Buffer. For Intracellular staining, the cells were incubated with anti- FoxP3 (PE) and anti-IL-17 (PE) at 4°Cfor 30 min in the dark. Each tube was added 1X Permeabilization Buffer with 1500 r/min centrifugation for 5 minutes and suspended in 200µl of 1X Permeabilization Buffer. Data were analyzed using the FACS Diva software (BD Biosciences, San Diego, CA).

***Detection of IL-17 serum levels in rats in each group by ELISA***

The level of cytokine in serum was measured by ELISA assay using a commercially available IL-17 ELISA kit in accordance with the manufacturer's instructions (Peters *et al*., 2011).

***Pathological sections by H&E staining***

The left anklebone from rats placed in 10% neutral formalin fixed solution for 24 hours. Then, placed in 10% EDTA for decalcification, vertically cut the anklebone after successful decalcification, embedded in paraffin, and the anklebone was cut into slices (4µm). The slices were treated by H&E staining to observe the inflammation in the joint. Histological scores were assessed according to a semi-quantitative method (Jianjian *et al*., 2014).

***Pathological sections by immunohistochemistry***

The sections were routinely dew axed in water. The sections were placed in citric acid in a pressure cooker for antigen retrieval. The sections were incubated in antigen with 0.3% $H_2O_2$ for 3 minutes, and washed with PBS 3 times for 3 minutes. A correct amount of primary detection antibody (IL-17 and FoxP3 antibodies) was added at 37°C for 1 hour. Subsequently, the sections were incubated with the biotin-labeled secondary antibody (sheep anti-rabbit IgG) at 37°C for 15 minutes, and washed with PBS 3 times. DAB coloration was performed, and water flushing was carried out after the right coloration. The sections were stained with hematoxylin, dehydrated, and gum mounted. For immunohistochemical evaluation, sections were stained with Foxp3 and IL-17 staining (Chung *et al*., 2012). The counting of the $Foxp3^+$ and $IL\text{-}17^+$ cells was performed by a pathologist and the Laboratory of Pathology (Guilin Medical College, China).

**Table 2**: Flow cytometry of spleen cells obtained from rats sacrificed 10 weeks after induction of CIA

| Group | Number of cases | Treg cells | Treg cell apoptosis | Th17 cells | Th17 cell apoptosis |
|---|---|---|---|---|---|
| Control | 10 | 0.45±0.07 | 9.50±0.28 | 0.40±0.00 | 16.85±11.10 |
| CIA | 10 | 0.82±0.23 | 5.90±3.50 | 0.98±1.30 | 7.74±6.77 |
| CIA + MTX + Pred | 10 | 1.48±0.53[a,b] | 4.48±2.18[a,b] | 0.60±0.35[a,b] | 6.38±5.40[a,b] |
| CIA + MTX + HCQ + SSZ | 10 | 1.88±0.72[a] | 3.24±1.82[a] | 0.70±0.20[a] | 4.40±1.98[a] |
| CIA + MTX + ETN | 10 | 1.68±0.89[a,b] | 2.63±2.33[a,b] | 0.68±0.35[a] | 4.88±3.12[a] |

Note: [a]*P*<0.05, *vs.* the CIA group; [b]*P*<0.05, *vs.* the MTX +HCQ + SSZ group

**Table 3**: Flow cytometry of spleen cells obtained from rats sacrificed 21 weeks after induction of CIA

| Group | Number of cases | Treg cells | Treg cell apoptosis | Th17 cells | Th17 cell apoptosis |
|---|---|---|---|---|---|
| Control | 10 | 0.98±0.36 | 18.72±13.07 | 0.58±0.29 | 43.60±11.65 |
| CIA | 10 | 0.93±0.42 | 20.94±14.23 | 1.20±1.04 | 48.01±17.19 |
| CIA + MTX + Pred | 10 | 1.04±0.29[a] | 21.86±8.39[a,b] | 0.63±2.50[a,b] | 16.94±5.47[a] |
| CIA + MTX + HCQ + SSZ | 10 | 0.94±0.24[a] | 16.94±7.41[a] | 0.26±0.16[a] | 16.73±3.60[a] |
| CIA + MTX + ETN | 10 | 1.40±0.65[a,b] | 16.94±13.33[a] | 0.59±0.49[a,b] | 26.07±14.59[a,b] |

Note: [a]*P*<0.05, *vs.* the CIA group; [b]*P*<0.05, *vs.* the MTX + HCQ + SSZ group

***Ethical approval***

The ethical approval was taken from The Animal Care and Welfare Committee of Guilin Medical University Hospital, Guilin, China (2014104001).

## STATISTICAL ANALYSIS

All data are expressed as mean ± SD, and all statistical analyses were performed using the statistical software SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The indicators at different time points were analyzed by ANOVA. If it was statistically significant, the SNK-q test was used for comparisons between the two groups, with a p-value <0.05 indicative of statistical significance.

## RESULTS

***CIA rats and treatment groups***

As shown in fig. 1A, 9 days after primary immunization, CIA rats revealed arthritis symptoms, which mainly included paw and ankle swelling. After 10 weeks, the joints were deformed, and some of the seriously rats could not crawl. The 'AI' reached a peak at week 6.

As shown in fig. 1B, after immunizations, 'AI' in the MTX + Pred group was significantly lower than the other treatment groups, followed by 'AI' in the MTX + ETN group (P<0.05). The weight of rats in the MTX + HCQ + SSZ group significantly increased with the progress of treatment over time. fig. 1C shows the weight of rats in the MTX + Pred group was inferior to that in the other treatment groups after 15 weeks (fig. 1C). In fig. 1D, the reduction of swelling degree in the MTX + Pred treatment was significantly reduced after 6 weeks.

***CIA rat limb joints by X-rays***

fig. 2A shows that compared to the control rats, narrowing of joint space and bony erosions were observed in the CIA rats, which had an aggravating tendency with the development of the disease. fig. 2B and C shows that compared to the CIA group, the MTX + Pred group significantly improved in the structural changes, followed by the MTX + ETN group. For radiographic evaluation, CIA rats receiving the MTX + Pred treatment displayed significant decreases in erosion and JSN scores as compared with the other treatment groups (P<0.01). The positive effect of the MTX + Pred treatment was superior to the MTX + HCQ + SSZ and MTX + ETN treatments.

***ELISA analysis***

As can be seen in table 1, it is obvious that the IL-17 serum level in the MTX + Pred group was inferior to that in the CIA and the MTX + HCQ + SSZ groups (*P*<0.01). However, IL-17 serum level in the MTX + Pred treatment was equivalent to that in the MTX + ETN treatment with no statistically significant difference. Detection of the amount of Th17 and Tregs and apoptosis from the spleens of CIA rats by flow cytometry

As can be seen in fig. 3, table 2 and 3, the Tregs population significantly increased in the MTX + HCQ + SSZ treatment, followed by the MTX + Pred and MTX + TNFi treatments. The Tregs population in the MTX + Pred treatment was closely to the MTX + TNFi treatment. The Th17 cells population in the MTX + HCQ + SSZ treatment was lower than that in the other treatments. However, the Th17 cells population in the MTX + Pred treatment was closer to the control group.

***Evaluation by H&E staining and immunohistochemical staining***

As shown in fig. 4A and 4B, the pathological changes in the MTX + Pred treatment significantly improved at 10 weeks. After 21 weeks, the MTX+HCQ+SSZ treatment changed more degrees than the other combination therapies. The improvement in MTX + Pred treatment was superior to the other combination therapies. The results of semi-quantified analysis indicated that the MTX + Pred treatment significantly decreased destruction of ankle joint ($P$<0.05, *vs.* the CIA group). The efficacy of the MTX + Pred treatment was superior to the MTX + HCQ + SSZ and MTX + ETN treatments.

Fig. 4C shows immunohistochemical analysis revealed that the expression of IL-17 in the MTX + Pred group was much lower than that in the CIA group and the other treatment groups ($P$<0.05). fig. 4D shows Foxp3 expression was higher in the MTX + HCQ + SSZ group, followed by the MTX + Pred group. The expression of Foxp3 in the MTX + Pred group was similar to that in the MTX + ETN group, compared to the MTX+HCQ+SSZ group.

## DISCUSSION

This study investigated the MTX + Pred treatment significantly increased DMARDs effects and improved the therapeutic potential of drugs. We compared radiographic and cytologic characteristics on CIA rats received the combination therapies with Pred, DMARDs and TNFi in MTX, respectively. Notably, the MTX + Pred treatment showed a marked decrease in the extent of the symptoms associated with RA.

In a blind trial, O'Dell *et al*. have indicated that approximately 70 % of the RA patients needed to require combination therapy (Smolen and Steiner, 2003). The current paradigm of RA focuses on features at earlier stages of disease and the effect of DMARDs therapy according to the ACR/EULAR criteria (Aletaha *et al*., 2010). Moreover, Todoerti *et al*. have shown low-dose Pred plus DMARDs therapy in early RA induces higher and earlier disease activity control, which supplies a higher probability for a more stable clinical remission over time (Todoerti *et al*., 2010). Recently, most scholars consider that glucocorticoid plays a role of the "bridge" in RA, which can suppress RA progression of radiographic joint damage and should not be used as monotherapy (Jong *et al*., 2004; Santiago *et al*., 2015; Lim *et al*., 2016; Wasko *et al*., 2016). However, the study that low doses glucocorticoid reached DMARDs was unclear. Thus, we performed these relevant experimental studies to confirm the effect of the MTX + Pred treatment.

Therefore we analyzed the amount of Tregs and Th17 cells, which may declare the efficacy in the MTX + Pred treatment to a great extent (Furst and Emery, 2014). The MTX + Pred treatment decreased the progression of disease, which up regulated Tregs expression and down regulated Th17 expression. The release of IL-17 from the MTX + Pred treatment was obviously inferior to this from the MTX + DMARDs treatment by ELISA. Meanwhile, the SHS score in the MTX + Pred treatment was lower than the MTX + DMARDs and MTX + TNFi treatments. The results of histological score assessment showed that the MTX + Pred treatment significantly improved, compared with the MTX + DMARDs and MTX + TNFi treatments. So the efficacy of the MTX + Pred treatment was much higher than in the MTX +DMARDs and MTX + TNFi treatments.

The combination therapies can be adopted by Tregs to secrete transforming growth factor-β1 (TGF-β); as well as to promote positive feedback on Tregs differentiation, inhibit Th17 cells and osteoclast differentiation and maturation and reduce inflammatory cytokine production (Ding *et al*., 2012). Tregs up-regulated the expression of osteoprotegerin. TGF-β and IL-6 synergistically inhibited nuclear factor κB ligand and colony stimulating factors, thereby inhibiting bone damage caused by Th17 cells. Therefore, the MTX + Pred treatment obviously improved erosion and JSN by X-ray, followed by MTX + TNFi treatment.

Therefore, we believed that a low dose of Pred had DMARDs effects, which was proved by the amount of Tregs and Th17 cells and IL-17 serum level. Tregs and Th17 cells provided an experimental and theoretical basis from clinical treatment decision. However, its long-term application in the clinical treatment of RA needs to be verified through multi-center randomized controlled trials; and its side effects require more attention. Some limitations of our study are essential to further discussion. The animal model of CIA mimic is not completely mimic human arthritis, but it is a valuable model for understanding pathogenesis and developing drugs (Zaiss *et al*., 2007; Schett and Gravallese, 2012).

## CONCLUSION

The main finding from the results presented here is that low doses of Pred displayed strong effect on RA therapy, particularly for the frequency and function of Th17 cells and Tregs, the scores of X-rays, H&E staining and immunohisto chemistry. Interestingly, IL17 serum level in the MTX + Pred treatment was equivalent to that in the MTX + ETN treatment. The level of IL17 was considered important to improve acute and chronic inflammation. The combination Pred in MTX not only decrease the RA symptom, but also have the same effect on the combination ETN in MTX, which is superior to the combination DMARDs in MTX. Unfortunately, we did not observe any effect on patients with different degrees in RA. We do not intend to change the use of currently available drug regiments for treatment of RA, but complement them through investigate Th17 cells and Tregs which have effect on RA microenvironment. The

efficacy of the MTX + Pred treatment was superior to the MTX + DMARDs treatment, equivalent to the MTX + TNFi treatment and Pred had the similar effect as DMARDs over time. Thus, the MTX + Pred treatment probably is a novel method in clinical treatment.

## ACKNOWLEDGMENTS

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