

## Original Article

# Metformin regulates Th17/Treg cell balance and reduces hyperplastic synovium via activating AMPK and inhibiting mTOR in a collagen-induced arthritis rat model

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**Abstract:** Objectives: This study aimed to determine the therapeutic effects of metformin and its mechanism in a collagen-induced arthritis (CIA) rat model. Methods: CIA rats were induced by bovine collagen II. CIA rats were treated with 30, 100, 300 mg/kg metformin or saline (Met-30 mg/kg, Met-100 mg/kg, Met-300 mg/kg and CIA groups, respectively). Detection of right hind paw volume and histological examination of the synovium were performed. Serum pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-17) were measured by ELISA. Flow cytometry was done to determine the percentages of CD4<sup>+</sup> ROR $\gamma$ t<sup>+</sup> IL-17A<sup>+</sup> and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T-cells in spleen lymphocytes. Western blotting was used to assess the expression of AMPK-mTOR in the spleen and Bax, Bcl2, Caspase-3, AMPK-mTOR expression in the synovium. Results: Metformin ameliorated both hind paw volume and hyperplastic synovium in CIA rats, decreased the serum levels of pro-inflammatory cytokines, and regulated the percentages of Th17, Treg cells in the spleens in a dose-dependent manner. Metformin down-regulated Bcl2 and up-regulated Bax, cleaved-Caspase-3 in the synovium, furthermore, activated AMPK and inhibited mTOR in the spleen and synovium. Conclusions: Metformin has therapeutic effects on CIA, which is ascribed to the regulation of Th17 and Treg cell balance and the reduction of hyperplastic synovium via activating AMPK and inhibiting mTOR. Metformin may be a potential therapeutic candidate for the treatment of rheumatoid arthritis.

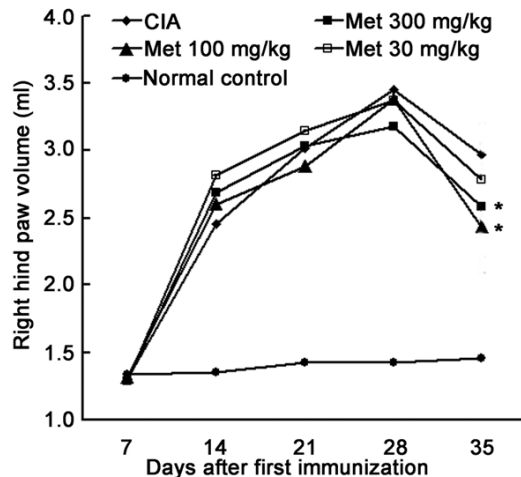
**Keywords:** Collagen-induced arthritis, metformin, AMPK-mTOR, TH17, Treg

## Introduction

Rheumatoid arthritis (RA) is a chronic, multisystem inflammatory disorder of unknown etiology. It is characterized by polyarthritis and systemic inflammation [1]. The inflammation in the hyperplastic synovium can destruct the structure of affected joints [2]. Although the exact molecular mechanism underlying the pathogenesis of RA remains unknown, it is suggested that T helper cell (Th) 17 plays a central role [3]. Interleukin (IL)-17, mostly secreted by Th17, has synergistic effects with tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and IL-6 in cases of RA [3]. Conversely, regulatory T (Treg) cell has been found to possess anti-inflammatory activity, but its role in the RA is poorly understood. Th17 and Treg cells often act with each other, and

can be transformed mutually depending the cytokine milieu [4]. These suggest that the balance between Th17 and Treg cells plays an important role in the development and the outcome of RA. Therefore, to regulate the balance of Th17/Treg may be a novel strategy in the treatment of RA.

RA is closely related to diabetes mellitus (DM). Available studies show the incidence of DM in RA patients is higher than in healthy controls [5, 6]. Secondary DM usually develops as a result of excessive use of corticosteroid in RA patients, and both disorders may cause cardiovascular complications. Metformin, a traditional antidiabetic medication, exerts glucose lowering effects by activating AMP-activated protein kinase (AMPK), a critical enzyme involved in the



**Figure 1.** Therapeutic effects of metformin on right hind paw volume of CIA rats. Rats in 30 mg/kg (n=6), 100 mg/kg (n=6), 300 mg/kg (n=6) metformin groups and saline (n=6) groups were orally fed with metformin or saline once daily (\* $P < 0.05$  vs CIA group).

lipid and glucose metabolism [7]. In addition to the antidiabetic effect, metformin has been shown to inhibit LPS-induced inflammation by suppress NF- $\kappa$ B production, which is also regulated by AMPK [8]. In addition, metformin activated AMPK may inhibit mammalian target of rapamycin (mTOR), which then regulate the differentiation of T cells *in vitro* and *in vivo* [9-11]. In particular, AMPK has been reported to be associated with the inhibition of TH17 cells through suppressing the phosphorylation of mTOR and its downstream signal transducers, suggesting the therapeutic potential of AMPK agonists.

Besides the imbalance between Th17 cells and Treg cells is responsible for the chronic inflammation in RA, synovium hyperplasia also plays a central role in the joint destruction [2]. It has been reported that rapamycin, an inhibitor of mTOR, is able to significantly reduce fibroblast-like synovial cell invasion in RA via suppressing mTOR signaling pathway [12].

These regulatory effects of AMPK on the inflammation, immune and fibroblast-like synovial cells have prompted the investigation on the effects of metformin on experimental arthritis [13, 14]. This study aimed to determine the therapeutic effects of metformin and its mechanism in a collagen-induced arthritis (CIA) rat model.

## Materials and methods

### *Immunity of animals and treatment with metformin*

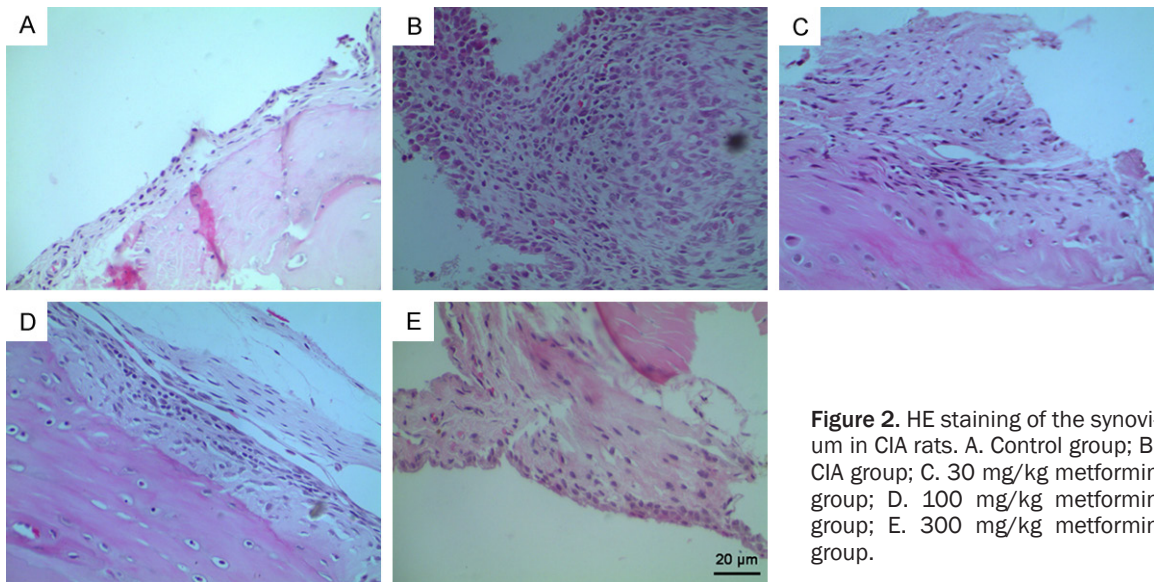
This study has been approved by the Ethics Committee of the First Hospital of Jiaying. Specific pathogen-free male Wistar rats weighing 140-160 g (Slac Laboratory Animal, Shanghai) were given *ad libitum* access to food and water. Animals were housed at constant temperature ( $25 \pm 2^\circ\text{C}$ ). Forty Wistar rats were randomly divided into 5 groups (CIA group, Met-30 mg/kg group, Met-100 mg/kg group, Met-300 mg/kg group and control group). Rats were intravenously immunized with 150  $\mu\text{g}$  of bovine CII (Chondrex, Redmond, USA) in complete Freund's adjuvant (Chondrex). Then, 100  $\mu\text{g}$  of bovine CII in incomplete Freund's adjuvant was intravenously injected on day 10 to induce CIA. CIA rats were orally fed with metformin at 30 mg/kg, 100 mg/kg and 300 mg/kg body weight in CIA groups or saline in control group starting from day 7 after first immunization [14]. In control group, saline was used instead of antibodies. The clinical severity of CIA was graded on a 0-4 scale as follows [13]: 0, normal; 1, swelling of the ankle or wrist, or limited to digits; 2, swelling of multiple sites in ankle, wrist or toes; 3, swelling of the entire paw; 4, maximal swelling. Each limb was graded by an observer in a blind manner. As a result, the maximum score for each animal was 16. On day 14 after first immunization, CIA was successfully induced in 6 rats in each group according to arthritis index greater than six. The rats were sacrificed on 35 days after first immunization.

### *Hind paw volume*

To determine the clinical therapeutic effects of metformin, the upper portion of rat right ankle joint was marked on the day of first immunization. Drainage method was used to measure the right hind paw volume once weekly.

### *Histological examination of the synovium*

After sacrificing, the synovium was collected from left ankle joint, fixed in 10% formalin, embedded in paraffin and cut into sections, followed by hematoxylin-eosin (H&E) staining for histological examination.



**Figure 2.** HE staining of the synovium in CIA rats. A. Control group; B. CIA group; C. 30 mg/kg metformin group; D. 100 mg/kg metformin group; E. 300 mg/kg metformin group.

## Measurement of serum cytokines

Blood samples were collected from orbital venous plexus on Days 35 after first immunization and centrifuged. The serum was harvested. Enzyme linked immunosorbent assay (ELISA) kits from RayBiotech (Norcross, GA, USA) were used to detect the levels of cytokines (IL-1 $\beta$ , IL-6, IL-17 and TNF- $\alpha$ ) in the serum according to the manufacturer's instructions. All specimens were assayed in duplicate. Standard curves were delineated and used to calculate the concentrations of cytokines in the serum.

## Lymphocytes isolation and flow cytometry

Lymphocytes were isolated from the spleen of experimental rats. To examine Treg cells,  $5 \times 10^5$  cells were first stained with APC-conjugated anti-rat CD4 (Ebioscience) and PE-conjugated anti-rat CD25 (Ebioscience), then with intracellular FITC-conjugated anti-rat Foxp3 (Ebioscience) according to the manufacturer's instructions at 2-8°C for 40-50 min in dark. For the detection of Th17 cells, cells were first stained with FITC-conjugated anti-rat CD4, intracellular FITC-conjugated anti-rat ROR $\gamma$ t (Abcam) and PE-conjugated anti-rat IL-17A (Ebioscience).

## Western blotting

The proteins were extracted from spleens and synoviums. The concentration of total proteins was evaluated using Bradford assay (KeyGEN

BioTECH). Then, proteins were loaded for SDS-PAGE, and then transferred onto PVDF membranes. The membrane was blocked in 5% skim milk and then incubated with following primary monoclonal antibodies (mAbs): anti-AMPK $\alpha$  (Abcam) and phospho-AMPK $\alpha$  (Thr172) rabbit mAb (Abcam) and anti-mTOR rabbit mAb (Abcam), anti-phosphor-mTOR (Ser2448) rabbit mAb (Abcam), anti-cleaved-Caspase-3 rabbit mAbs (Abcam), anti-Bax (KeyGEN BioTECH), anti-Bcl2 (KeyGEN BioTECH) and anti- $\beta$ -action (KeyGEN BioTECH). After washing with PBS, the membrane was incubated with the primary antibodies overnight at 4°C, followed by treatment with secondary antibody (goat anti-rabbit IgG-HRP; KeyGEN BioTECH). Data collected from Western blotting were analyzed with G: BOX chemiXR5 and Gel-Pro32.

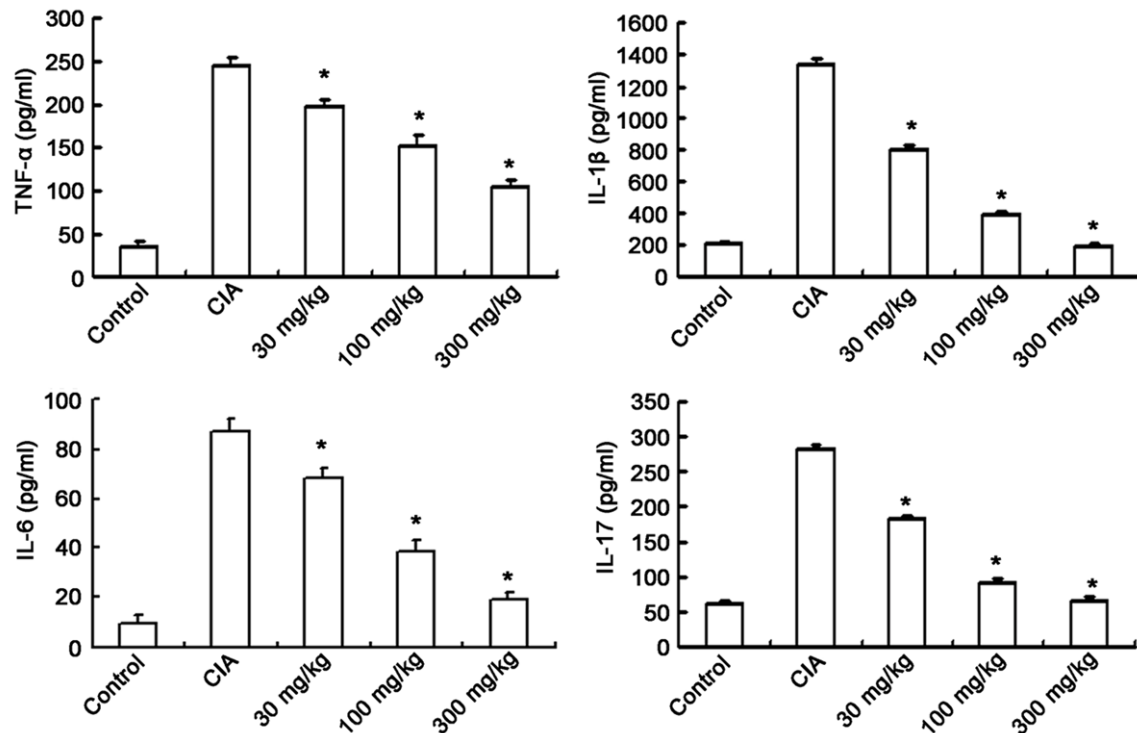
## Statistical analysis

All the data are expressed as mean  $\pm$  standard deviation (SD), and statistical analysis was performed with SPSS version 20.0. Comparisons were done with one way analysis of variance (ANOVA) followed by post hoc test. A value of  $P < 0.05$  was considered statistically significant.

## Results

### Metformin ameliorates joints swelling and synovium hyperplasia

To investigate the therapeutic effects of metformin on arthritis, CIA rats were orally fed with



**Figure 3.** Therapeutic effects of metformin on serum pro-inflammatory cytokines. TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-17 were measured by using ELISA on day 35 after first immunization. Data are expressed as mean  $\pm$  SD (n=6/group) (\*P<0.05 vs CIA group).

either metformin at different doses or saline beginning on day 7 after first immunization. In 100 mg/kg metformin and 300 mg/kg metformin groups, the right hind paw volume reduced significantly as compared to saline treated group (**Figure 1**). HE staining showed that synovium hyperplasia was also significantly attenuated after metformin treatment (**Figure 2**). These results showed that metformin has therapeutic effects on CIA.

#### Serum levels of pro-inflammatory cytokines

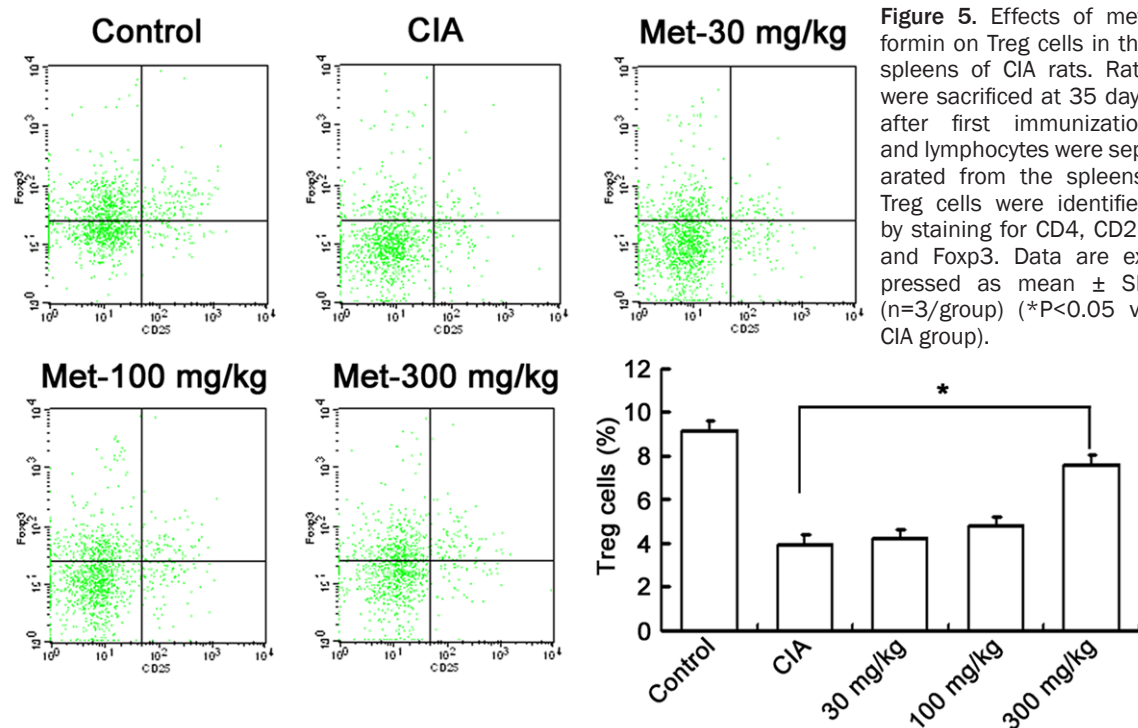
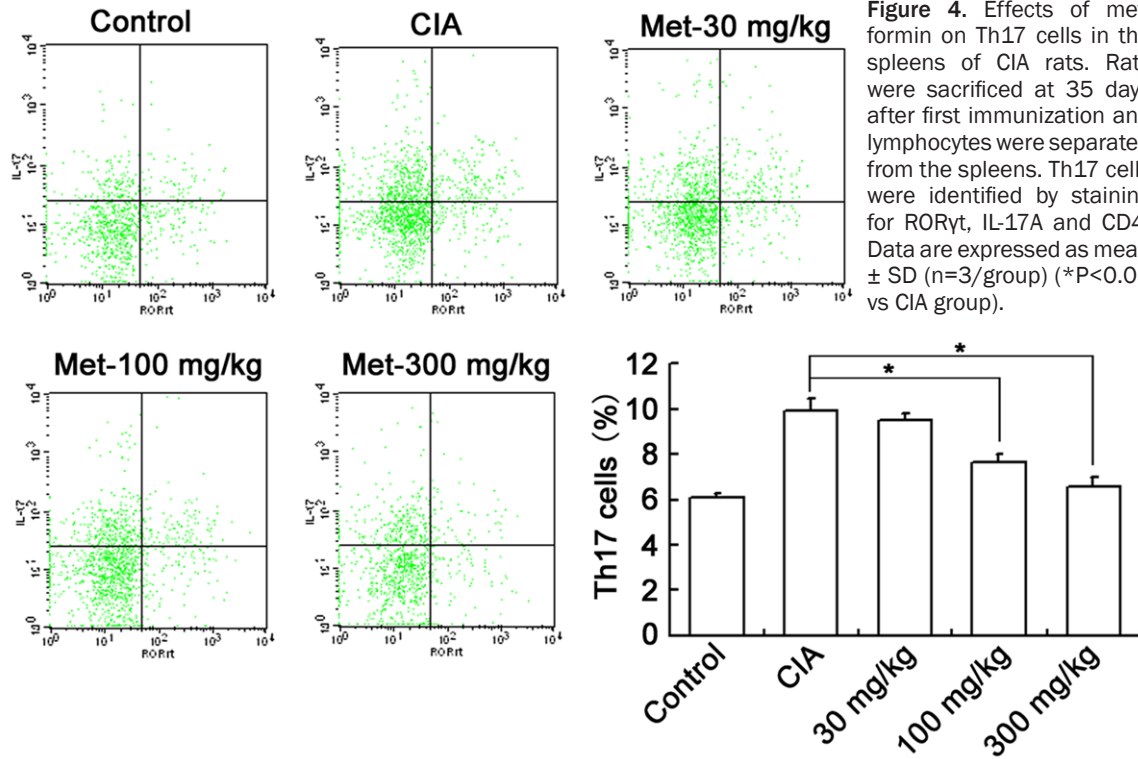
The contents of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-17) in the serum of CIA rats increased significantly as compared to normal rats. To investigate whether metformin inhibited their contents, blood samples were collected on day 35 after first immunization, and the serum levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-17 were measured by ELISA. The saline treated CIA rats showed higher TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-17 levels than the metformin treated rats, and the serum contents of pro-inflammatory cytokines were the lowest in 300 mg/kg metformin group. In addition, metformin re-

duced pro-inflammatory cytokines in a dose-dependent manner (**Figure 3**). These results suggest that metformin may ameliorate CIA through decreasing the serum pro-inflammatory cytokines.

#### Metformin regulates Th17/Treg balance in the spleen

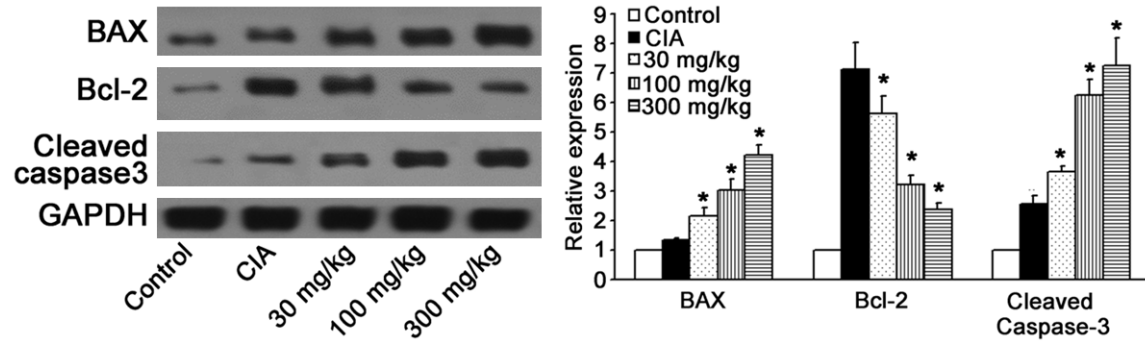
Th17 and Treg cells in the spleens were detected by flow cytometry at 35 days after first immunization. Th17 cells were identified by staining for ROR $\gamma$ t, IL-17A and CD4. Metformin reduced the number of CD4<sup>+</sup> ROR $\gamma$ t<sup>+</sup> IL-17A<sup>+</sup> T-cells in the spleens in a dose-dependent manner (**Figure 4**). This decrease in 100 mg/kg metformin group and 300 mg/kg metformin group was significantly higher than in saline treated rats with CIA. Treg cells were identified by staining for CD4, CD25 and Foxp3. Metformin enhanced the number of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T-cells in the spleens in a dose-dependent manner (**Figure 5**). This enhancement was significantly higher in 300 mg/kg metformin group than in saline treated rats with CIA. The ratio of TH17/Treg in 300 mg/kg metformin group was



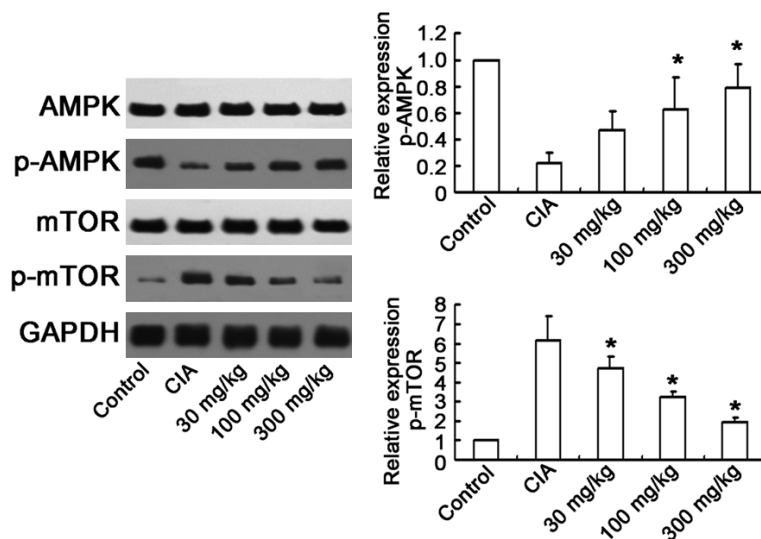


similar to controls. The differentiation of T cells and downstream characteristic cytokines play an important role in the pathogenesis of CIA

and RA in patients. Therefore, our results suggest that metformin may regulate Th17/Treg cells balance, and then inhibit the production of



**Figure 6.** Expression of BAX, Bcl-2 and Cleaved Caspase-3 in synoviums. Synoviums were processed for Western blotting for BAX, Bcl2 and Cleaved Caspase-3. Data are expressed as mean  $\pm$  SD. \*P<0.05 vs CIA group.



**Figure 7.** Expression of AMPK-mTOR in the spleen lymphocytes. Cells were obtained from the spleens, and subjected to Western blotting for AMPK, phospho-AMPK (p-AMPK), mTOR, and phospho-mTOR (p-mTOR). Data are expressed as mean  $\pm$  SD. \*P<0.05 vs CIA group.

pro-inflammatory cytokines to attenuate autoimmune arthritis.

#### *Metformin regulates the expression of Bax, Bcl2 and cleaved-caspase-3 in the synovium*

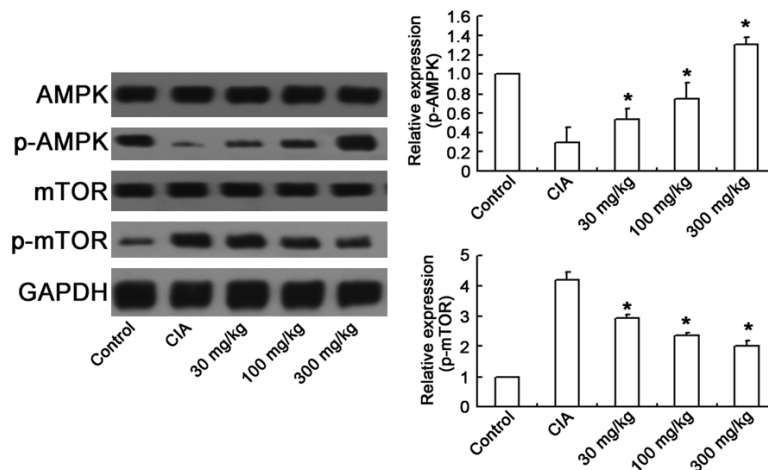
Bax and Bcl2, two apoptosis related proteins, play important roles in the cell death. The balance between Bax and Bcl2 contributes to the activation of caspase-3, an executive protein in cell apoptosis. To investigate the mechanism underlying metformin induced amelioration of synovium hyperplasia, the expression of Bax, Bcl2 and cleaved-Caspase-3 in the synoviums was determined by Western blotting. Our results showed that anti-apoptotic protein Bcl-2

significantly reduced and pro-apoptotic protein Bax and cleaved-Caspase-3 markedly increased in the synoviums of metformin treated CIA rats as compared to saline treated CIA rats (Figure 6). These suggest that metformin induced amelioration of synovium hyperplasia is related to its regulation on the Bax, Bcl2 and Caspase-3 expression.

#### *Metformin regulates Th17/Treg balance and ameliorates synovium hyperplasia through AMPK-mTOR pathway*

Metformin exerts its pharmacologic effects by activating AMPK. mTOR, inhibited

by AMPK activation, is involved in the differentiation of T cells and might ameliorate synovium hyperplasia. In the present study, Western Blotting was employed to detect the protein expression of phosphorylated AMPK and mTOR in the spleens and synoviums. As shown in Figures 7 and 8, compared with saline treated CIA rats, the expression of phosphorylated AMPK increased significantly in the spleens and synoviums of metformin treated CIA rats. In addition, metformin also inhibited the phosphorylation of mTOR in a dose-dependent manner. These findings suggest that metformin regulates Th17 and Treg cells differentiation and ameliorates synovium hyperplasia through activating AMPK and inhibiting mTOR.



**Figure 8.** Expression of AMPK-mTOR in the synoviums. Synoviums were processed for Western blotting for AMPK, phospho-AMPK (p-AMPK), mTOR, and phospho-mTOR (p-mTOR). Data are expressed as mean  $\pm$  SD. \* $P < 0.05$  vs CIA group.

## Discussion

Metformin, an AMPK activator and a traditional anti-diabetic drug, has been confirmed to possess anti-inflammatory activity in recent years [8, 15]. A recent study shows that increased AMPK phosphorylation is able to attenuate LPS-induced inflammatory responses via inhibiting NF- $\kappa$ B activation in murine macrophages [8]. Our results revealed that metformin significantly reduced the right hind paw volume on day 35 after first immunization and decreased the serum pro-inflammation cytokines in CIA rats. In 300 mg/kg metformin group, the serum levels of IL-1 $\beta$ , and IL-17 were similar to those in normal control group. These results suggest that metformin has anti-inflammatory activity. Although the therapeutic effect of metformin on the hind paw volume was weaker than that of traditional disease-modifying anti-rheumatic drugs, available studies showed the incidence of DM in RA patients is higher than in controls [5, 6]. Secondary DM usually develops as a result of excessive use of corticosteroid in RA patients. Thus, we speculate that metformin used in the treatment of RA may also improve DM in RA patients.

Although the exact molecular mechanism underlying the pathogenesis of RA remains unknown, evidence suggests that abnormal differentiation and functions of TH17 and Treg cells play important roles [3]. Our results

revealed that the number of Th17 and Treg cells in saline treated CIA rats was significantly different from that in controls. It further confirmed the role of TH17 and Treg cell differentiation in the pathogenesis of RA. There is evidence showing that metformin may improve CIA via regulating Th17 cells [13]. Metformin-activated AMPK and mTOR, its downstream molecule, seems to be involved in this regulation. Furthermore, similar to the findings from the study of Son et al [14], our results also revealed the differentiation of Th17 and Treg cells was regulated by metformin.

The number of Th17 and Treg cells and the ratio of TH17/Treg were comparable between normal control group and 300 mg/kg group. AMPK is a cellular energy detector involved in the regulation of glucose, fat and protein metabolism [7]. Results findings suggest that Th17 cells and Treg cells use different energy sources [16]. For instance, Treg cells have a high lipid oxidation rate while Th17 cells are highly glycolytic. AMPK, activated by metformin, enhances Treg cells by promoting mitochondrial oxidative metabolism. mTOR, inhibited by AMPK, enhances the differentiation of Th17 cells through promoting the glycolytic pathway. Our results revealed that metformin enhanced the expression of phosphorylated AMPK while inhibited phosphorylated mTOR in the spleens. Therefore, we postulate that metformin induced AMPK activation and mTOR inhibition are able to ameliorate CIA by regulating Th17 and Treg cells balance.

Synovium hyperplasia plays a central role in the joint destruction, and the hyperplasia of fibroblast-like synovial cells in RA is similar to that of cancer cells [17, 18]. The hyperplasia of cancer cells may be suppressed via metformin induced inhibition of protein synthesis by activating AMPK and inhibiting mTOR [19]. A recent study reports that rapamycin, an inhibitor of mTOR, significantly reduces the invasion of fibroblast-like synovial cells in RA via suppressing mTOR signaling pathway [10]. HE staining in our study

showed that synovium hyperplasia was significantly attenuated in metformin treated rats, accompanied by enhanced expression of phosphorylated AMPK while reduced expression of phosphorylated mTOR in the synoviums. Furthermore, our findings demonstrated that metformin significantly down-regulated the anti-apoptotic protein Bcl-2 expression and up-regulated the pro-apoptotic proteins Bax and cleaved-Caspase-3 expression in the synoviums of CIA rats. The expressions of several apoptotic genes, such as Bax and Bcl2, which are regulated by extracellular factors including mTOR, are involved in the cell death [20, 21]. When the ratio of Bax/Bcl2 increases, the pro-apoptotic proteins are released from mitochondria to activate caspases and induce apoptosis [22]. Therefore, we speculate that metformin induced AMPK activation and mTOR inhibition are effective to ameliorate CIA by regulating synovium hyperplasia through down-regulating Bcl2 expression and up-regulating Bax and cleaved-Caspase-3 expression.

### Conclusion

In conclusion, our results indicate that metformin is able to ameliorate CIA by regulating Th17/Treg cells balance, activating AMPK and inhibiting mTOR to suppress synovium hyperplasia. In RA patients with concomitant DM, metformin is a preferred drug due to its glucose lowering and anti-arthritis properties.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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