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The impact of Clonorchis sinensis infection on immune response in mice with type II collagen-induced arthritis

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Abstract

Background Clonorchis sinensis infection could trigger strong immune responses in mice and human. However, whether the C.sinensis infection has an impact on arthritis is unknown. Here we investigated the effect of C.sinensis infection on type II collagen-induced arthritis in BALB/c mice.

Results The mice were firstly infected with 45 C.sinensis metacercariae by oral gavage. Four weeks later, the arthritis in mice was induced by type II collagen. Joint inflammation with severe redness and swelling in hind paws were observed in type II collagen-induced arthritis (CIA) mice. In addition, the physical activity was significantly reduced, but the respiratory exchange ratio was increased in CIA mice. Compared with CIA mice, C.sinensis infection could increase the severity of arthritis in CIA mice, based on the results of disease score and pathological changes. Compared to CIA mice, increased neutrophils and Ly6C hi monocytes, decreased B cells and CD4+T cells were found in C.sinensis infected CIA mice. Besides these, C.sinensis infected mice also displayed significant higher levels of serum IL-4 and IL-17 than those in CIA mice. Taken together, our data suggest that C.sinensis infection have a bad effect on arthritis, and could induce the abnormality of immune response in mice with CIA.

Background

Rheumatoid arthritis (RA) is a kind of autoimmune disease, which is characterized with chronic joint inflammation, synovial membranes proliferation and vasculogenesis, and could lead to the cartilage and bone destruction [1]. It is estimated that, 24.5 million people are effected by RA[2], and the published reports have been shown that the immune responses mediated by neutrophils, T cells, B cells, macrophages and associated cytokines, including TNF-α, interleukin (IL)-6, IL-17 and IL-1, play important roles in RA [3]. Therefore, further understanding the factors that could have an effect on the immune response in RA maybe help us find potential strategies for treatment of the disease.

It is well known that parasitic infection is capable of inducing the host immune response towards a strong type 2 immune response, which is could be induced by the activation of Th2 cells with the secretion of IL-4, IL-5 and IL-13 [4,5]. Besides these, T helper 17 (Th17) cells, Th9 and T regulatory (Treg) cells also participate in the immune response during parasitic infection [6]. Furthermore, current studies suggest that early infection of certain parasites may prevent the development of antoimmune diseases, including allergic disease and RA [7], and the mechanisms are associated with immune responses mediated by parasite infection. For example, it has been shown that *Schistosoma japonicum* infection and *Trichinella spiralis* derived antigen could reduce the severity of collagen-induced arthritis [8], via immune reactions related to IL-4 and Treg Cells mediated by parasite antigens in CIA mice.

Clonorchiasis, induced by *clonorchis sinensis*, is widely prevalent in Asian region, including China, Korea, Vietnam and the far east regions of Russia. Just like other helminthes, *clonorchis sinensis* infection could stimulate immune response with characterized by Th2-dominated response and the imbalance of Treg/Th17 [9,10]. So far, the effect of clonorchis sinensis on arthritis has not been reported. The main aim

of the current study was to assess the effect of *clonorchis sinensis* infection on immune response on collagen-induced arthritis in mice.

Results

Mice infected with *C.sinensis* metacercariae

As shown in Figure 1, the CIA mice was successfully infected with *C.sinensis*, and the parasite infection could induce the pathological damage of liver in CIA mice. In portal areas of liver of the *C.sinensis* infected CIA mice, accompanied with hepatic fibrosis, the extensive inflammatory cell infiltrations were observed.

C.sinensis infection increases the joint swelling and clinical score.

Around 4 weeks after type-Ⅱ collagen immunization, the mice, in CIA groups and *C.sinensis*+CIA groups, developed the signs of arthritis. Analysis of paws/joints showed that the paws and knees of *C.sinensis* infected arthritic mice were more swollen than these mice in CIA group. Moreover, the clinical scores of *C.sinensis* infected arthritic mice on day 24, 27, 30, 33 and 39, were significantly higher than CIA group (Figure2).

The changes of ambulatory activity and indirect calorimetry after C.sinensis infection in CIA mice.

CIA could induce the change of the joint swelling in mice, and the motion of joints could be altered. In the present study, we detected the physical activity of the mice in three groups. The physical activity of the mice in both *C.sinensis*+CIA group and CIA group were significantly reduced than that those mice in control group. But there was no difference in *C.sinensis*+CIA and CIA group (Figure3A). We further analyzed the respiratory exchange ratio (RER) of three groups, and the result showed that the RER of mice in *C.sinensis*+CIA group and CIA group were higher than that in mice from the control group (Figure3B). No difference of RER were found in mice between *C.sinensis*+CIA group and CIA group (Figure3B).

C.sinensis infection alters the humoral immune response in CIA mice

To estimate the influence of *C.sinensis* infection on the humoral anti-collagen response in mice, we compared the levels of anti-collagen IgG in the serum in the three groups. As shown in Figure 3, compared with the mice in the control group, a significant increased level of anti-collagen IgG was presented in mice in both *C.sinensis*+CIA and CIA group. But there was no difference in mice in *C.sinensis*+CIA group and CIA group (Figure4).

The changes of immune cells after *C.sinensis* infection in CIA mice.

Neutrophils, a member of innate immune system, can be attracted and quickly recruited into sites of infection, play important role during autoimmunity, injury and chronic disease . It's well known that neutrophils play an essential role in rheumatoid arthritis[13]. We assessed the effect of infection with *C.sinensis* on neutrophils, monocytes, T cells and B cells in CIA mice. As shown in Figure5, the percentage of neutrophils and $CD11b^{+}Ly6c^{hi}$monocytes in mice from *C.sinensis*+CIA group were significantly higher than those in mice in CIA group and control group. However, compared with CIA group and control group, the percentages of CD4+T cells and B cells were lower in *C.sinensis*+CIA group.

The changes of inflammatory cytokines after *C.sinensis* Infection in CIA mice

In order to explore the effect on of *C.sinensis* infection in CIA, the cytokines of IL-4, IL-6, IFN-γ, IL-17 and TNF-α in serum in the mice in three groups were detected. As shown in figure 5, the levels of IL-4 and IL-17 were significantly higher in *C.sinensis*+CIA group than CIA group and control group. Although compared to control group, higher levels of IL-6, IFN-γ and TNF-α were found in *C.sinensis*+CIA group and CIA group, there was no difference in IL-6, IFN-γ and TNF-α in *C.sinensis*+CIA group and CIA group.

Discussion

RA is an inflammatory disorder that mainly affects the joints and synovial tissue and leads to the pain and physical disability. The pathogenesis of RA is complex and the etiology is unknown. So far, despite many avenues, including antirheumatic drugs and immune-modulate therapies, have pursued, there is still no effective, safe and affordable treatments. Previous studies have indicated that certain parasitic infections could reduce the incidence and severity scores of RA [14]. In this study, we evaluated whether *C.sinensis* infection have an effect on RA by using CIA mice models.

To confirm mice were successfully infected with *C.sinensis*, the livers of mice were obtained for pathological analysis. The infiltration of several inflammatory cells was found in hepatic portal areas. In addition, the destruction of liver tissue structure caused by fibroblast proliferation and collagen deposition, and the destruction of bile duct structure that characterized with cholangiocyte hyperplasia, narrowing of bile duct lumen and periductal fibrosis were found (Figure1). These results were consistent with the typical pathological of *C.sinensis* infection as described in our previous research [15].

Current studies show that different parasitic infections have distinct effects on rheumatism. For example, *Schistosoma japonicum* infection could reduce the severity of rheumatic diseases in CIA mice [7]. However, Graepel et al. reported that when mice infected with *Hymenolepis diminuta*, the arthritis was exaggerated, and the mice had more severe clinical symptoms [16]. We assessed the effect of *C.sinensis*

infection on CIA in mice. Obvious joint swelling and significant clinical score were found in CIA mice in the study. In addition, with pathological analysis, compared to CIA mice, many inflammatory cells infiltration around the joints, fibroplastic proliferation and pannus information were found in *C.sinensis*+CIA mice (Figure2). Based on the results of disease score and pathological changes, our data indicated that *C.sinensis* infection could increase the severity of the disease.

Collagen is an important component of cartilage. In rheumatoid arthritis, type-Ⅱ collagen is a critical autoantigen, and collagen-specific antibodies are frequently found in RA patients [17]. The anti-type-Ⅱ collagen antibodies could form immune complexes and activate complement, meanwhile monocyte, granulocyte, and T cells are attracted to the joints [18]. In the study, we found that *C.sinensis* infection in mice could not change the levels of anti-type-Ⅱ collagen antibody in CIA model.

With joint swelling, pain and the development of arthritis, the physical activities of *C.sinensis*+CIA mice and CIA mice were found to be seriously limited. However, the mice in CIA group and *C.sinensis*+CIA group had the same performance. Interestingly, the RER in CIA group and *C.sinensis*+CIA group were higher than that control group. It was reported that mice with chronic inflammation have increased RER [19], and the mice could alter their metabolism as a response to colonic inflammation. In the study, no difference of RER were observed in *C.sinensis*+CIA mice and CIA mice. Taken together, these results suggested that CIA could decrease the physical activities but increase RER, while *C.sinensis* infection have no significant impacts on the physical activities as well as RER in the mice model we built.

Neutrophil could be enrolled to the site of inflammation and play an important role during infection, autoimmunity and chronic disease. In RA patients, 90% cells in the synovial fluid are neutrophils [20]. Furthermore, these sells can also be seen in the pannus and cartilage. It has been reported that neutrophil depletion can inhibit the development of arthritis [21-22]. LY6C^{hi} monocytes are recruited to the sites of inflammation, after extravasation, they can differentiate into macrophages and dendritic cells [23]. Ly6C^{hi} inflammatory monocytes make significant contribution during CIA development, and the number of Ly6C^{hi} monocytes is associated with the severity of disease and Ly6C^{hi} monocytes depleting can reduce the inflammation and bone erosion in CIA [24-26]. T cells and B cells also play important roles in RA. In the early stage of arthritis, antigen-presenting cells, including dendritic cells, activated B cells and macrophages could present antigens to T cells. Then CD4^{+}T cells secrete IL-2 and IFN-γ in synovial membrane [27]. Furthermore, B cells can produce antibodies, autoantibodies and cytokines, and contribute to the pathogenesis of rheumatoid arthritis. Activation of T and B cells further causes the increased production of cytokines and chemokines, and results in the formation of feedback loops between T cells and B cells [28]. Besides, it is well known that helminth infection is typically characterized by inducing host adaptive immune towards type 2 immune response. The activation of Th2 cells can secrete IL-4 and IL-13 [29]. CD4+T cells play critical role in helminth infections, and CD4^{+}T-deficient mice would be incapable of stimulating anti-helminth immune response [30]. We are interested in detecting whether the *C.sinensis* infection have an impact on immune response mediated by neutrophils, monocytes ,T cells and B cells mediated in RA. Our data showed that the number of neutrophils and

LY6C^{hi} monocytes in both CIA group and *C.sinensis*+CIA group were higher than those in control group. Furthermore, compared to CIA group, the number of neutrophils and LY6C^{hi} monocytes were higher in *C.sinensis*+CIA group. In addition, compared with control group and CIA group, the number of B cells and CD4^{+}T cells in *C.sinensis*+CIA group were reduced. Taken together, these results suggested that *C.sinensis* infection could cause the dysfunction of immune response mediated by neutrophils, LY6C^{hi} monocytes, CD4^{+}T cells, and B cells in CIA.

Cytokines are usually secreted by immune cells, and contribute to the regulation of the activation, differentiation, migration and survival of host cells with various types *in vivo*. It has been shown that, several cytokines, including IL-6, TNF-α, IL-17 and IFN-γ play dominant roles in RA [31]. These cytokines could regulate immune response through complex signal pathways, and thus affect the pathological process of RA. In addition, blocking the function of TNF-α and IL-6 by monoclonal antibodies, could significantly reduce the pathobiology of RA[32,33]. Besides these, our research also found that, compared to control group, the cytokines, including IL-6, TNF-α and IFN-γ were elevated in both CIA group and *C.sinensis*+CIA group. But there was no difference between CIA group and *C.sinensis*+CIA group. These results indicated that *C.sinensis* infection have no significantly affect on expression of these three kinds of cytokines. In addition, the levels of IL-4 and IL-17 in *C.sinensis*+CIA group were found to be significantly higher than those in both CIA group and control group. These results suggested that the immune response in CIA could be influenced by *C.sinensis* infection, via increase the expression of IL-4 and IL-17 in mice.

Taken together, although it has been reported that the certain parasites, including *Schistosoma japonicum*, could significantly attenuate the clinical signs of arthritis in mice, and the associated mechanisms are related to immune reaction mediated by parasite infection [34,35].

Conclusions: In this study, our results indicated that *C.sinensis* can't ameliorate the symptom of arthritis. Furthermore, to some extent, *C.sinensis* can exacerbates an experimental model of arthritis, and the effect of *C.sinensis* infection on arthritis may be associated with the change of immune cells and associated cytokines.

Methods

The collection of *C.sinensis* metacercariae

Fresh fish infected with *C.sinensis* metacercariae were collected and transported from Guangxi Autonomous Region, People's Republic of China. There was no specific permission required during the collection.Then the fish were minced and digested with artificial gastric juice (0.7% pepsin in 1% HCL) at 37℃ for 12h, as described by Yan, et al [11]. The digested mixture was filtrated through 1000μm, 300μm and 106μm sieves. Then the metacercariae were identified and collected with dissecting microscope.

Animals

BALB/c mice (6-8week) were purchased from Shanghai Laboratory Animal Co., Ltd (SLAC, Shanghai, China). All mice were maintained in the SPF laboratory of Xuzhou Medical University. All animal experiments were approved by the Animal Care and Use Committee of Xuzhou Medical University(No. SCXK < SU > 2015–0030).

Induction and clinical assessment of arthritis

Mice were randomly divided into 3 groups as follows:) Control group (Control),) type II collagen-immunized group (CIA group),) *C.sinensis* infection and type II collagen-immunized group (*C.sinensis*+CIA group). In *C.sinensis*+CIA group, the mice were infected about 45 *C.sinensis* metacercariae by intragastric administration. Four weeks later, the mice in and groups were immunized with 100μg of bovine type- collagen in complete Freund's adjuvant intradermally on the back of the mice. Two weeks later, the second immunization with 100μg of type- collagen in incomplete Freund's adjuvant was performed. From 3 weeks post-immunization, the severity of arthritis was evaluated every 3 days. The arthritis score was quantified based on swelling and redness (graded from 0-4 for each paw; 0: normal; 1: slight swelling and redness; 2: moderate swelling and redness; 3: severe swelling and redness in large joints and moderate swelling and redness in small joints; 4: most severe swelling and redness in large joints and severe swelling and redness in small joints [12].

Joint histology

Mice were sacrificed on day 35 with carbon dioxide.The ankle joints were collected and fixed in 4% paraformaldehyde (PFA) for 1 week. Following fixation, the joints were decalcified in 12.5% EDTA2Na for 1 month, with the solution changed every 2 days. Next, the joint tissues were embedded and sectioned into 4 μm-thick slices. The cartilage destruction, vascular proliferation, inflammatory cell infiltration and synovial hyperplasia were assessed by hematoxylin-eosin and masson staining.

Detection of antibodies against type- collagen

Serum was collected from mice on day 35 after first type- collagen immunization. The level of anti-type- collagen IgG was detected by ELISA. In brief, 96-well ELISA plates were coated with type- collagen at 5μg/ml overnight. Serum sample was incubated and specific secondary antibodies were used. After enzymatic reaction, the absorbance was measured at a 450 nm wavelength.

Ambulatory activity and indirect calorimetry.

Physical activity and indirect calorimetry were assessed by Columbus Comprehensive Lab Animal Monitoring System (CLAMS, Columbus, USA). Briefly, mice were transferred into metabolic cages for 1 day of acclimation. Then the physical activity and respiratory exchange ratio (RER) were recorded every 5 or 6 min for 48 hours.

Flow cytometry

The number of neutrophils, B cells, and CD4+T cells were analyzed by flow cytometry. Briefly, peripheral blood mononuclear cells (PBMCs) from mice in different groups were stained with anti-mouse CD45, anti-mouse CD4, anti-mouse B220, anti-mouse CD11b, anti-mouse Ly6G, anti-mouse Ly6C for 30 minutes. After washing with PBS, the cells were analyzed with BD FACSCanto flow cytometer.

Cytometric Bead Array (CBA)

Serum was collected from mice on day 35 after first type- collagen immunization. The concentrations of IL-4, IL-6, IFN-γ, IL-17 and TNF-α were detected by CBA assay with BD FACSCanto flow cytometer, and the cytometric data were analyzed with FCAP Array software v 3.0 (BD Biosciences).

Statistical analysis

All data were analyzed by SPSS18.0 and presented as mean ± standard deviation. One-way ANOVA was performed to analyze the statistical significance.

Abbreviations

C.sinensis: Clonorchis sinensis CIA: collagen-induced arthritis

RA: Rheumatoid arthritis ELISA: enzyme linked immunosorbent assay

RER: respiratory exchange ratio CBA: Cytometric Bead Array

Declarations

Ethics approval and consent to participate

This study was conducted according to the Guidelines for the Laboratory Animal Use and Care Committee of the Ministry of Health, China and the Ethics Committee on Animal Research of Xuzhou Medical University (No. SCXK < SU > 2015–0030).

Consent for publication

Not Applicable.

Availability of data and materials

The data supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YL designed the study, performed experiments, and wrote the manuscript; YY and WC helped to collection of C.sinensis metacercariae ; SQ designed the experiments, collected the samples; HH detected cytokine; WP,QY analyzed the data; FK and CY helped to draft the manuscript; RT and KZ designed the research and revised the initial manuscript draft. All authors have read and approved the final manuscript.

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Figures

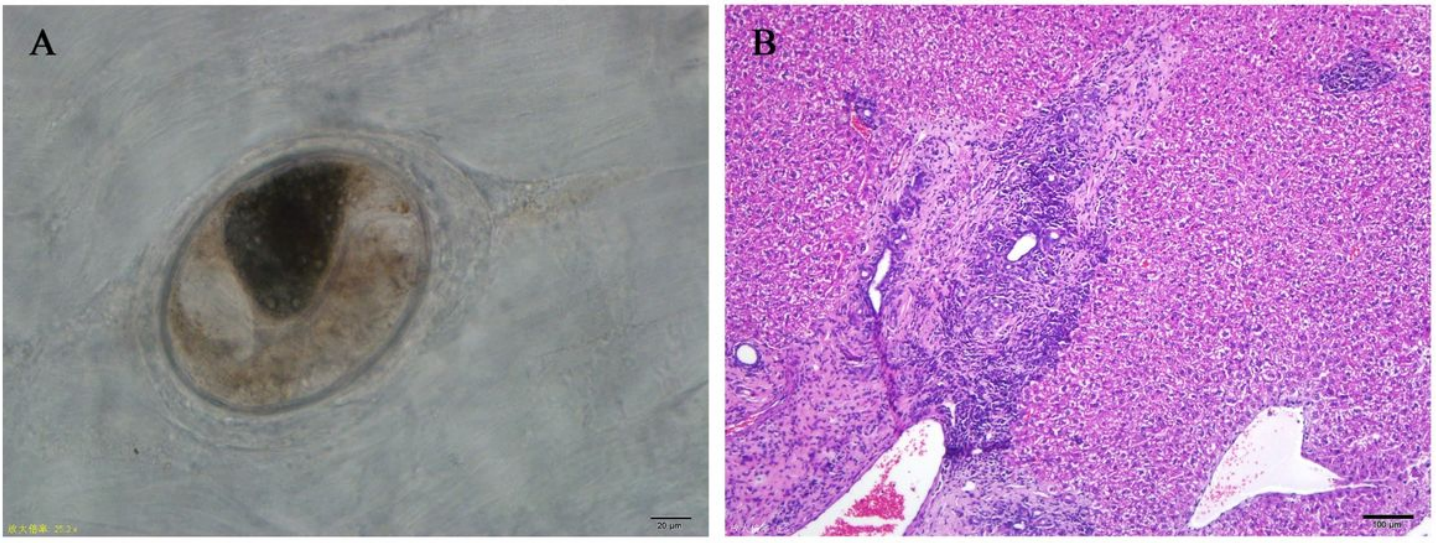

Figure 1

Mice infected with C.sinensis metacercariae. (A) C.sinensis metacercariae were collected from fish and examination by microscope. (B) Histological of liver tissues from C. sinensis-infected mice stained with hematoxylin and eosin (H&E). Histological structure of liver was destroyed. Massive inflammatory cells infiltrated around the portal area accompanied by collagen hyperplasia and cholangiocyte proliferation.

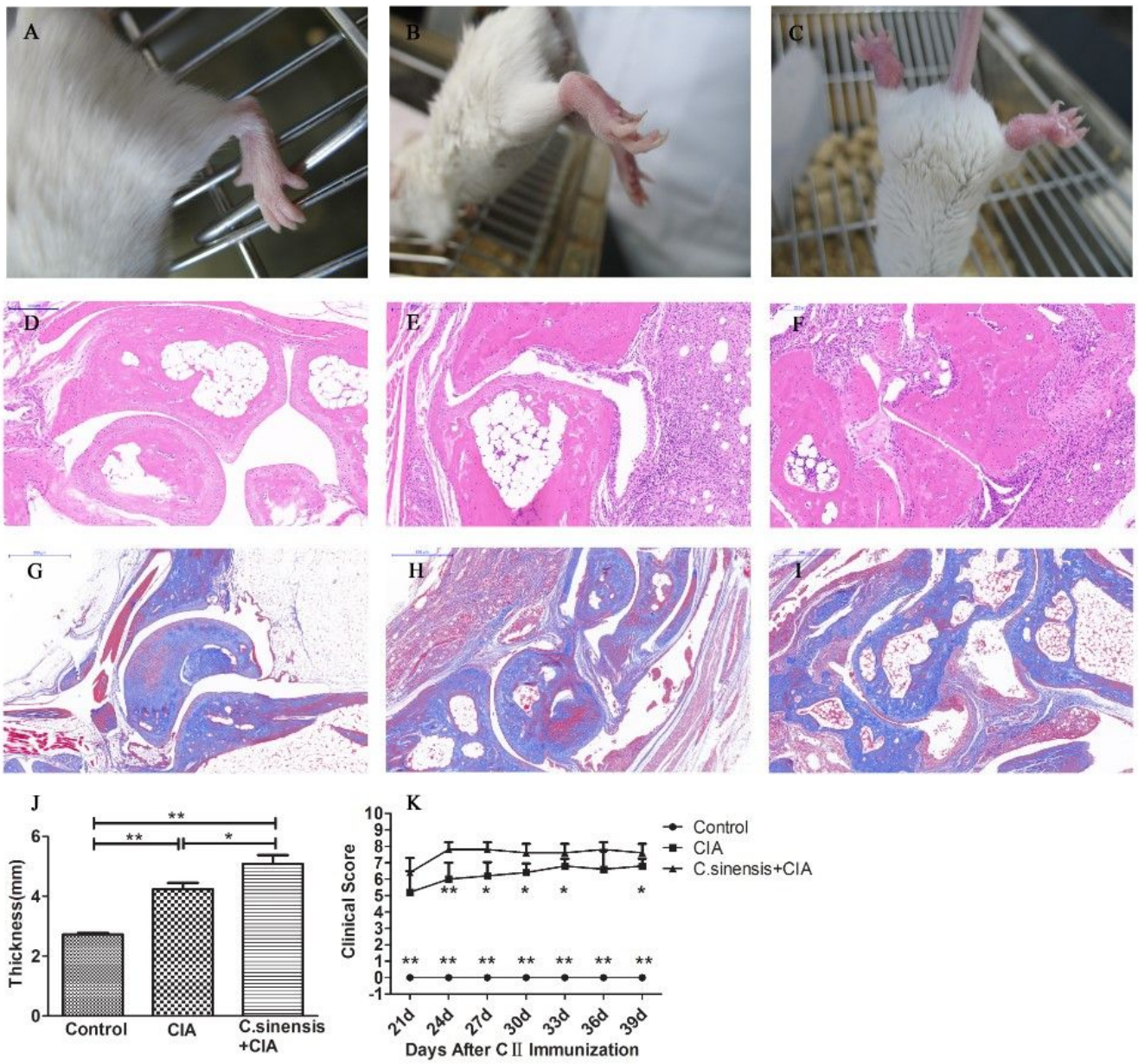

Figure 2

Effects of C.sinensis infection on the development of CIA in BALB/c Mice. (A-C) Representative images of arthritic limbs at day 39. (A) normal control mice,(B) CIA mice,(C) CIA and C.sinensis infection mice showing severe redness and swelling in both hind paws. (D-F) Images are H&E staining of ankle joints of mice. (D) normal control mice, (E) CIA mice, (F) CIA and C.sinensis infection mice showing inflammatory infiltration, synovial hyperplasia ,pannus formation and the destruction of joints. (G-I) Images are masson staing of ankle joints of mice. (G) Normal control mice, (H) CIA mice, (I) CIA and C.sinensis infection mice showing the proliferation of collagen fibers and cartilage destruction.(J-K)Data show the thickness of ankle joints and the total score of four limbs after C Ⅱ immunization (n=5/group). Asterisks mark statistically significant difference (*P<0.05,**P<0.01).

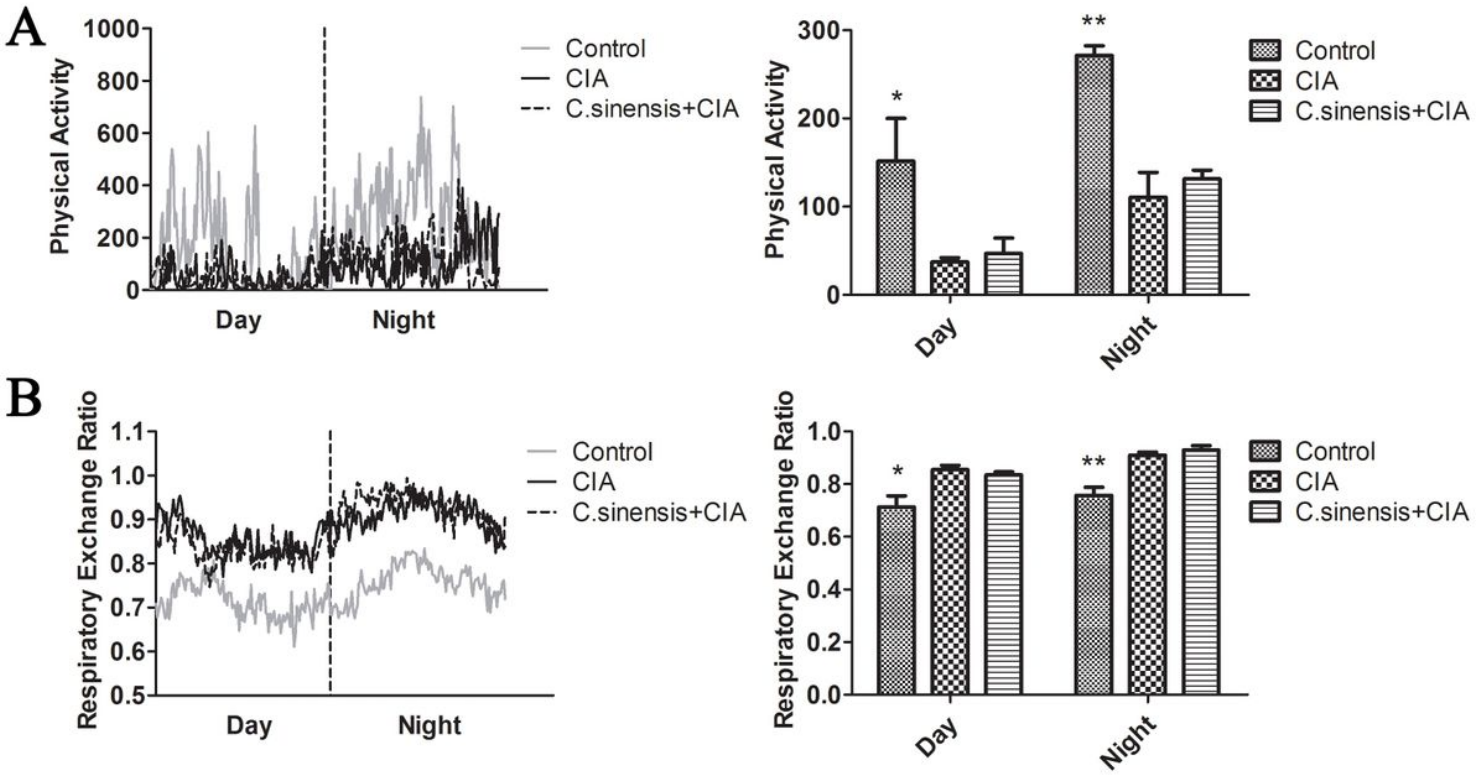

Figure 3

Mice were housed in automated metabolic cages. Physical activity (A), respiratory exchange ratio (B), were monitored.For bar graphs,data are shown as means+SEM (n=5); for line graphs,data are shown as the mean for each group (n=5) during a 24-hour cycle. Asterisks mark statistically significant difference (*P<0.05,**P<0.01).

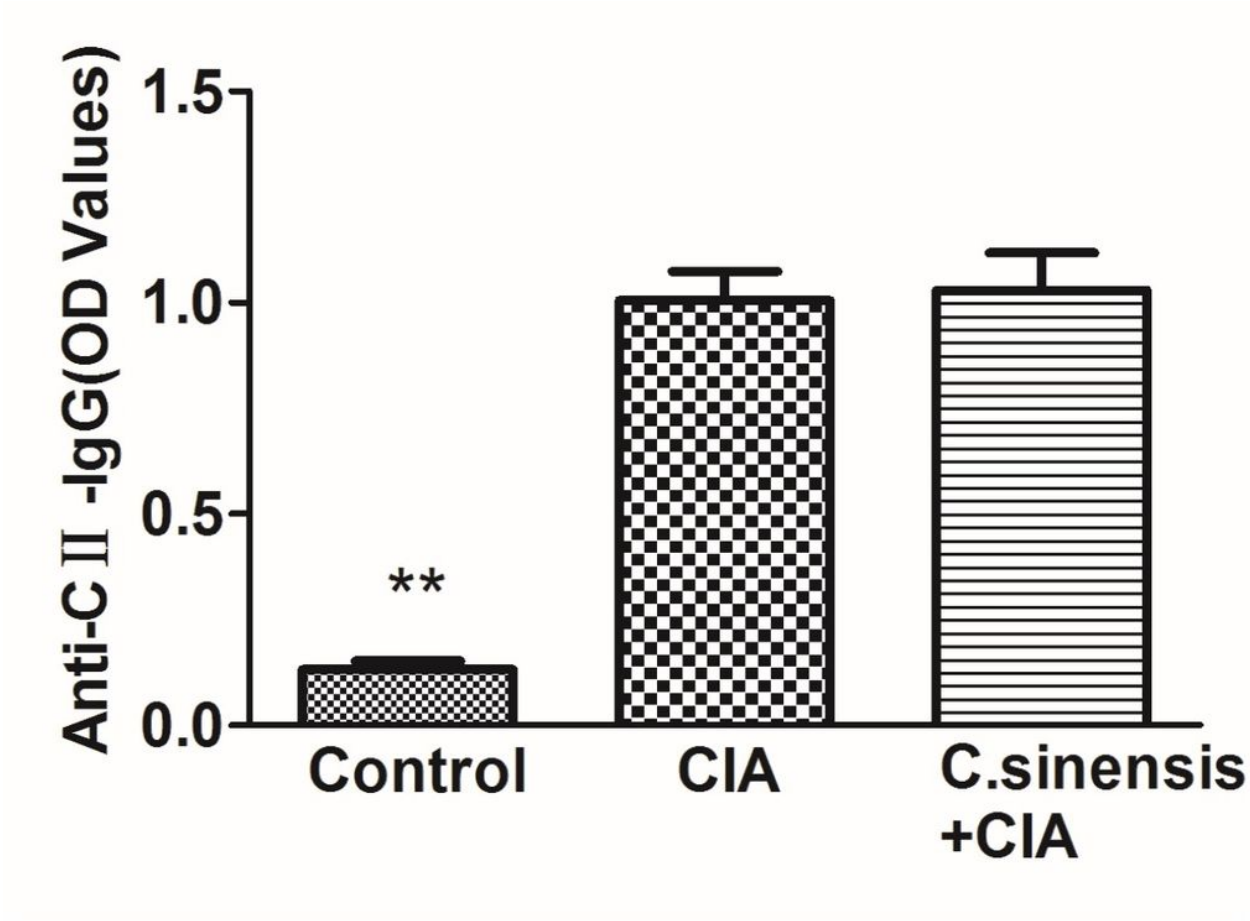

Figure 4

The anti-collagen IgG in the serum were tested by ELISA. Both C.sinensis+CIA group and CIA group have higher level of anti-collagen IgG(**P<0.01).But there was no difference between C.sinensis+CIA group and CIA group.

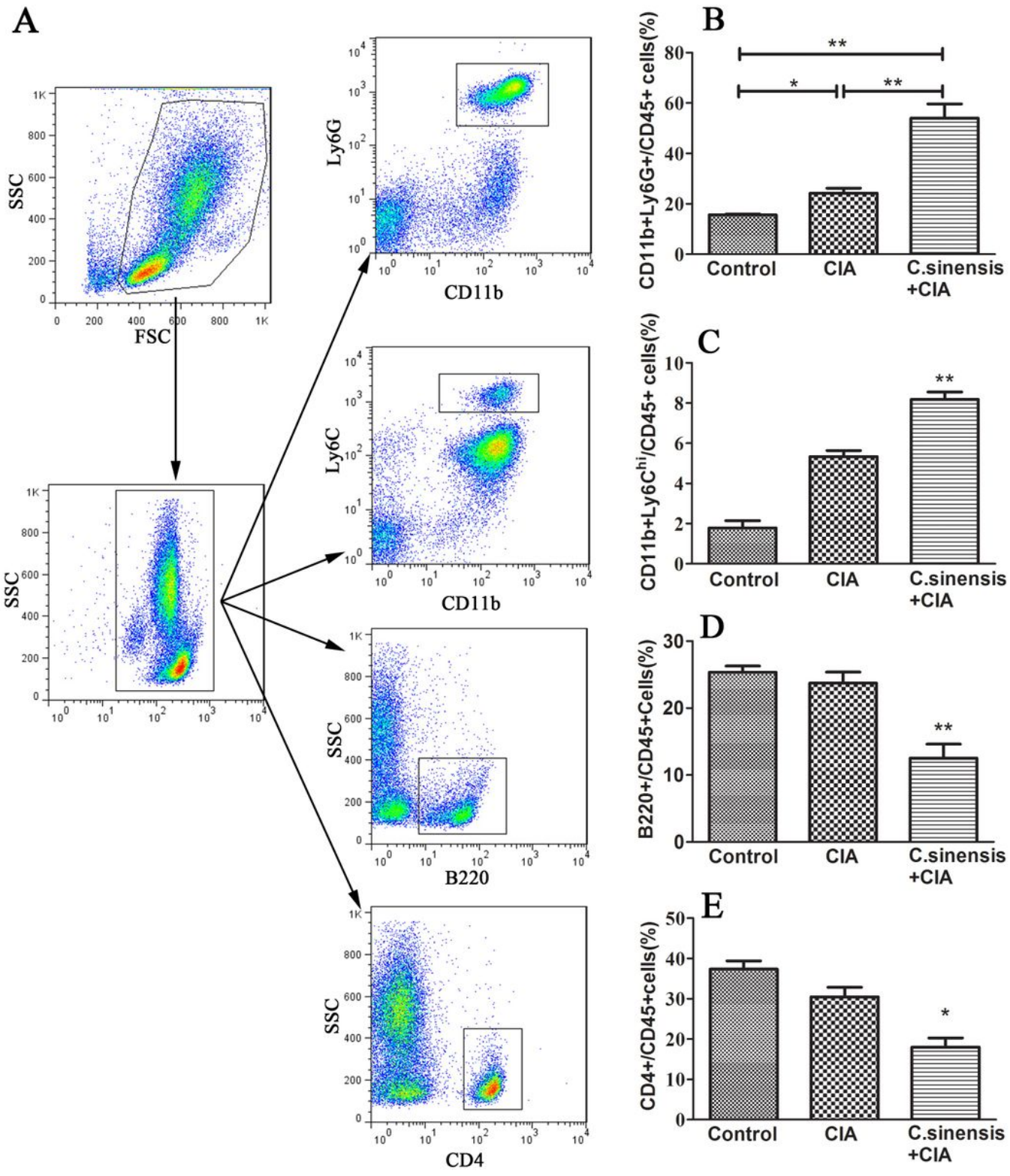

Figure 5

Effects of C.sinensis infection on immune cells. Peripheral blood were collected from mice. (A) Neutrophils, Ly6c+monocytes, B cells and CD4+T cells were analyzed by flow cytometry. (B, C) Compare to CIA group and control group, the percentage of neutrophils and Ly6chimonocytes were higher (**P<0.01). (D, E) However the percentage of B cells and CD4+T cells were lower than CIA group and control group (*P<0.05).

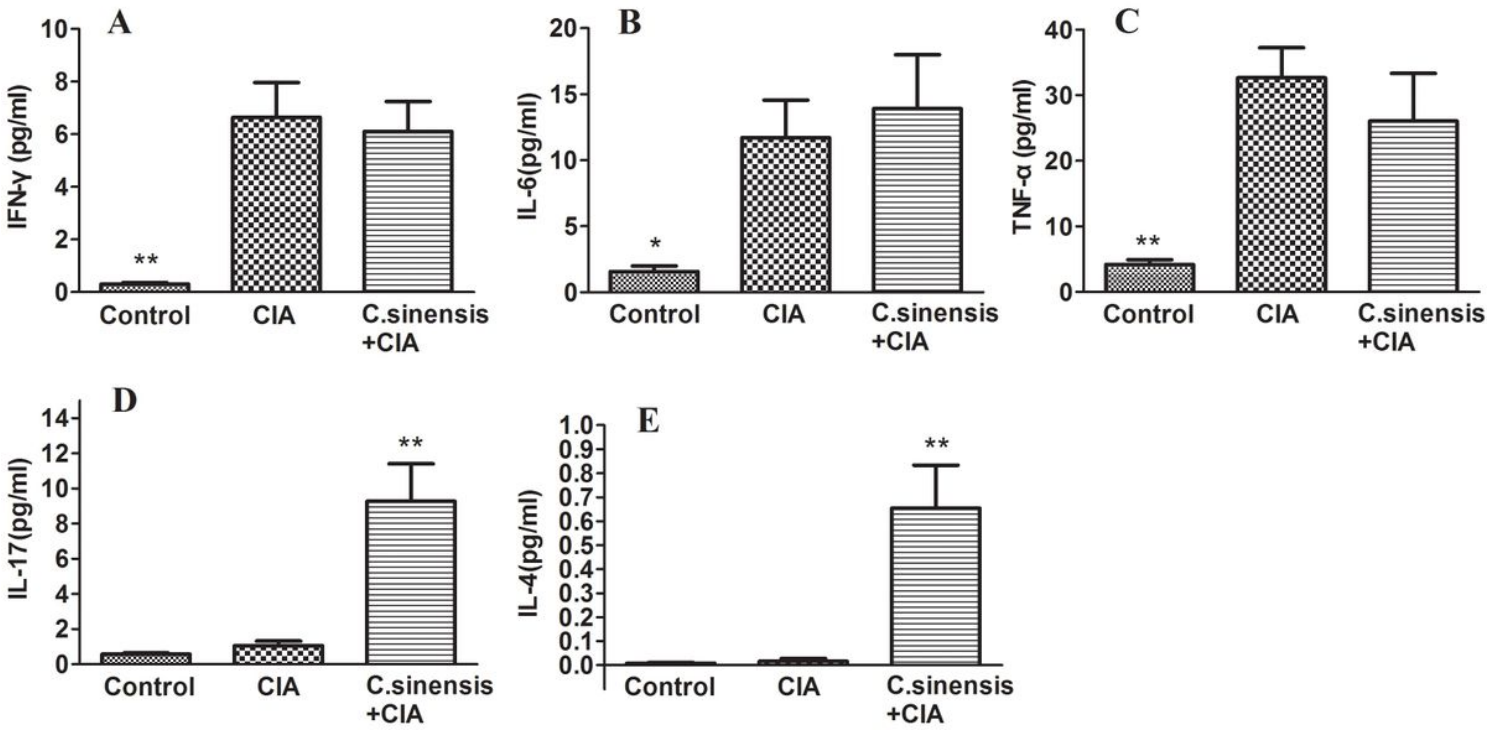

Figure 6

Effects of C.sinensis infection on systemic cytokines. Serum were collected and analyzed for cytokines by CBA as described in materials and methods. (A, B, C) Data show that both CIA group and C.sinensis+CIA group,the level of IFN-γ, IL-6 and TNF-α were higher than control group. But no difference were found in CIA group and C.sinensis+CIA group. However, C.sinensis infection can alter the level of IL-17 and IL-4. (D,E) The expression of IL-17 and IL-4 were significantly higher than CIA group. Asterisks mark statistically significant difference (*P<0.05,**P<0.01).

Supplementary Files

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