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IL-4 Can Inhibit IL-17 Production in Collagen Induced Arthritis

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ABSTRACT

Background: IL-4 is a cytokine that induces differentiation of naive helper T cells into Th2 cells. Once activated by IL-4, Th2 cells subsequently produce additional IL-4. **Objective:** To examine the effect of IL-4 on IL-17 production and its effect in Collagen-Induced Arthritis (CIA) mice. Method: In this study, a chicken collagen-II-induced experimental arthritis (CIA) model was used in DBA/1 mice to investigate the relationship between IL-4 and IL-17 as well as other inflammatory factors. On the 38th day after the mice were induced with CIA, the expression of IL-17 and IL-4 as well as IFN-γ and IL-13 in sera of the mice was measured by QRT-PCR and ELISA. **Result:** The result of QRT-PCR analysis of IL-17 and IL-4 mRNA levels in the spleen showed that IL-17 is increased significantly at the onset of CIA in the spleen ($p<0.01$). Meanwhile, IL-17 is generally reduced at the peak of CIA but IL-4 is increased significantly at this peak in the spleen ($p<0.05$) when the weight of the animal was taken into consideration. **Conclusion:** IL-4 can be involved in the production of IL-17 at especially the peak of CIA. These results imply that the inhibition of IL-17 may decrease the expression of IL-1β and IL-6 production which will result in the aggravation of arthritis.

Keywords: CIA, IL-4, IL-17

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic systemic autoimmune inflammatory disease characterized by systemic, multi-articular synovial arthritis (1). Type-II collagen is one of the primary structural components of hyaline cartilage and constitutes approximately half the dry weight of extracellular matrices (2). The animal model for the disease called the Collagen-induced arthritis (CIA). It is an experimental model sharing several clinical and pathological features with rheumatoid arthritis (RA). CIA has been used to study the pathogenesis of RA. The importance of T-cells in the pathogenesis of CIA and RA

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has been established and numerous studies have been performed to determine the cytokines and susceptibility factors involved in arthritis development (3,4). IL-4 is an anti-inflammatory cytokine which inhibits the production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and prostaglandin E2 (PGE2). Also, IL-4 up-regulates the expression of anti-inflammatory mediators such as IL-1 receptor antagonist and IL-1 type II receptor (5,6). Activated T-lymphocytes, basophiles and mast cells secrete IL-4 which regulates the proliferation of specific immune functions of B cells, macrophages, and hematopoietic cells (7). Interleukin 17 (IL-17) is a cytokine that acts as a potent mediator in delayed-type reactions by increasing chemokine production in various tissues to recruit monocytes and neutrophils to the site of inflammation, similar to Interferon gamma (8). IL-17 is produced by T helper cells and is induced by IL-23 which results in destructive tissue damage in delayed-type reactions. IL-17 functions as a proinflammatory cytokine that responds to the invasion of the immune system by extracellular pathogens and induces destruction of the pathogen's cellular matrix (9). IL-17 acts synergistically with tumor necrosis factor (TNF) and IL-1. Although there have been significant advances in understanding the development and maintenance of Th17 cells in vitro, the endogenous regulation of Th17 responses during the development of arthritis is still under investigation (10). Various antigenic stimuli can trigger IL-17 responses in vitro and not all of them will result in systemic or organ-specific autoimmunity in animal models. Our objective was to examine the regulatory effect of IL-4 and IL-13 on IL-17 production, thus revealing the possible mechanism of how IL-4 can inhibit the production of IL-17 in collagen-induced arthritis (CIA) in mice.

MATERIALS AND METHODS

Reagents. β-actin, IL-17, IL-4 and IL-13 primers were obtained from Sangon Company (Table 1). Primer express software (primer 5) was used to design primers from published cDNA sequences.

Cell Culture. Purified spleen cells (1×10^6 cells per well in a 24-well plate) were cultured for 3 days in complete RPMI-1640 medium (Gibco Invitrogen corporation, UK), supplemented with 20% fetal bovine serum and 1% streptomycin/penicillin.

Mice. DBA/1 mice were obtained from the Chinese Academy of Medical Sciences Shanghai Experimental Animal Center and maintained in a pathogen-free animal facility at Jiangsu University. All mice were kept in Jiangsu University animal center and their food and water changed twice a day.

Induction of CIA. Male DBA/1 mice at 7 weeks of age were immunized without anesthesia intra-dermally at the base of the tail with 200 μg of chicken collagen type II, dissolved in 1000 μl of 0.1 M acetic acid and mixed with an equal volume of CFA (Difco, MI, USA). A total of 100 μl was injected into each mouse. Three weeks later, the animals were re-immunized with C-II, emulsified in incomplete Freund's adjuvant (IFA) (Difco, MI, USA). The mice were observed three times per week for signs of joint inflammation and scored for clinical signs as follows: "0=normal"(with no erythema or swelling); "1=mild swelling" (confined to the ankle joint); "2=mild swelling" (extending from the ankle to the metatarsal or metacarpal joints); "3=moderate swelling" (extending from the ankle to the metatarsophalangeal or metacarpophalangeal joints); and "4=severe swelling" (extending from the ankle to the digits and resulting in ankylosis

and loss of joint movement). Mice were categorised into control group (5 mice), CIA group (8 Mice at the Onset of CIA and 7 mice at its peak).

Table 1. The primers used in the present study.

Gene	Sequence (5'-3')	Accession Number	Length (bp)
IL-13	5′-GAGCCCCGAGCTCTTCCCCA-3′ 5′-CGACCCCTCCACCTCGCAGA-3′	NM_019507	265
IFN-γ	5′-TATTCGGTAACTGACTTG-3′ 5′-AATCACATAGCCTTGC-3′	NM_000619	378
IL-4	5′-GGCAGCAAGGACGGCACCAA-3′ 5′-ATCGGTTGCGGCTGGTTCGG-3′	NM_011281	279
IL-17	5′-CAAGACTGAACACCGACTAAG-3′ 5′-TCTCCAAAGGAAGCCTGA-3′	NM_002190	231
β-actin	5'-TGGAATCCTGTGGCATCCATGAAA-3′ 5'-TAAAACGCAGCTCAGTAACAGTCC-3'	NM_001101	265

Histological Analysis. The knee joints were removed from the mice and fixed in 10% formalin and, after 4 days, was put in 5% formic acid for decalcification. Tissue sections were stained with hematoxylin for 5 minutes and washed for another 5 minutes.
Real Time PCR Analysis. Total RNA was isolated from the spleen (1×10^6 cells) and the knee synovium. The PCR reaction was performed with a 1 µL buffer, 1 µL of 5 mM dNTP, 2.5 µL of Taq polymerase, 1.2 µL of 50 mM $MgCl_2$, and 1 µL of each primer. The PCR condition was as follows: initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 40 minutes and a final elongation cycle at 72°C for 10 minutes (Corbett 6000 Real Time PCR). PCR products were run on 1% agarose gel (Shanghai Yito Enterprise Company-China) and stained with ethidium bromide. The electrophoresis bands were photographed using the gel doc system and analyzed by quantity gel analysis software.
Immunofluorescence Analysis. Mouse knee joints were sectioned at a thickness of 5 µm after being washed for 10 minutes in PBS, blocked with the mixture of 5% BSA and 0.1% Triton X for 30 minutes. A quantity of 50 µl of Fluoroisothiocyanate conjugated anti-mouse diluted IL-4 (1:50 in 5% BSA) was added to each section for 2 hours and washed with PBS four times. Fluoroisothiocyanate conjugated anti-mouse CD4 diluted 1:50 in 5% BSA was then added to the section for 2 hours and washed with PBS four

times. Hoechst dye was used to dye the nucleus. The results were observed with fluorescence microscopy (Olympus, USA).

ELISA (Enzyme -Linked Immunosorbent Assay). IL-4 levels in serum and spleen cell culture supernatants of the model and the control groups were then determined by IL-4 ELISA Kit (R&D system). IL-6 and IL-1β and IFN-γ levels were measured by ELISA kits (R&D system). We also used FLX-800 microplate reader from BioTekinstruments (Bitecs, USA).

Intracellular Staining. Spleen cells were obtained from the immunized mice and stimulated by PMA/ionomycin for 5 hours. Cells were stained with anti-CD4-FITC. Intracellular staining with antibodies against IL-17 (BD Biosciences) was performed and analyzed by flow cytometry.

Data Analysis. Data was summarized as mean ± SD. The statistical analysis of the results was performed by the Independent-Sample t test. Values for $p<0.05$ were considered significant.

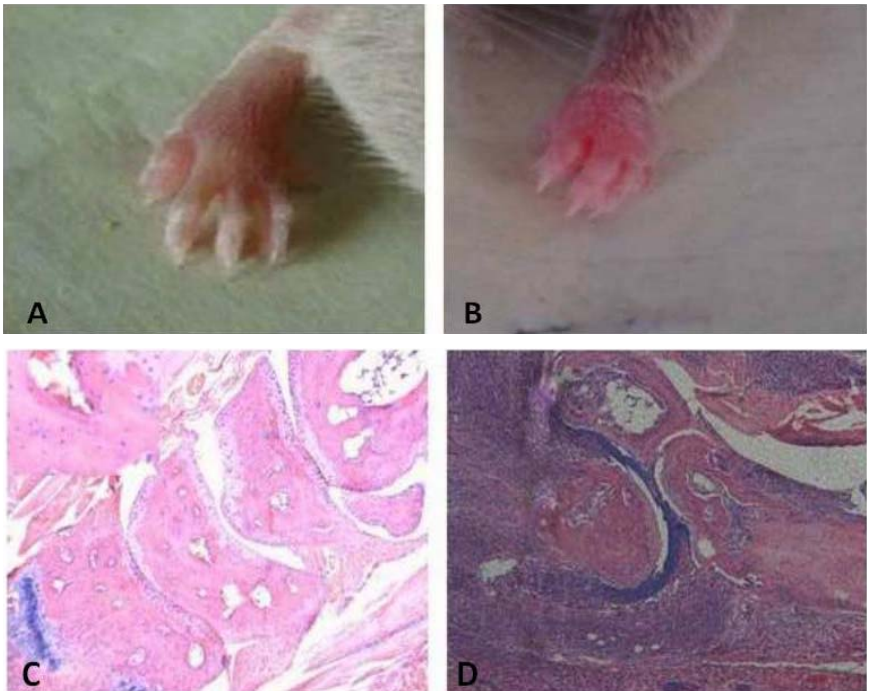

Figure 1. Joint swelling and pathological changes in CIA mouse. Onset of CIA is 34 days after the first immunization and the peak of CIA is 45 days after immuniation (A, B) with the development of CIA, the joint swelling increased. (C, D). The corresponding histological staining (HE 200 X) shows lymphocyte infiltration. At the peak of CIA, there are more lymphocytes in the knee joint cavity. There is a clear arthrofibrosis and a severe joint destruction at the peak of CIA.

RESULTS

Pathological Changes and Joint Swelling. At day 34 after the first immunization with collagen type-II, joint swelling was observed in the toe and foot palm of the mouse. The total period of this study was 45 days. Also, our result at the peak of CIA indicated that the swellings of the joints to be higher than in the onset group, with the control not

undergoing any change (Figures 1A and 1B). At 45 days after immunization, the arthritis began to resolve in the joint tissues.

Histological staining showed the presence of more lymphocytes in the hind leg knee in the CIA group compared with the control. All of the mice responded with arthritic scores >1 (Figures 1C and 1D).

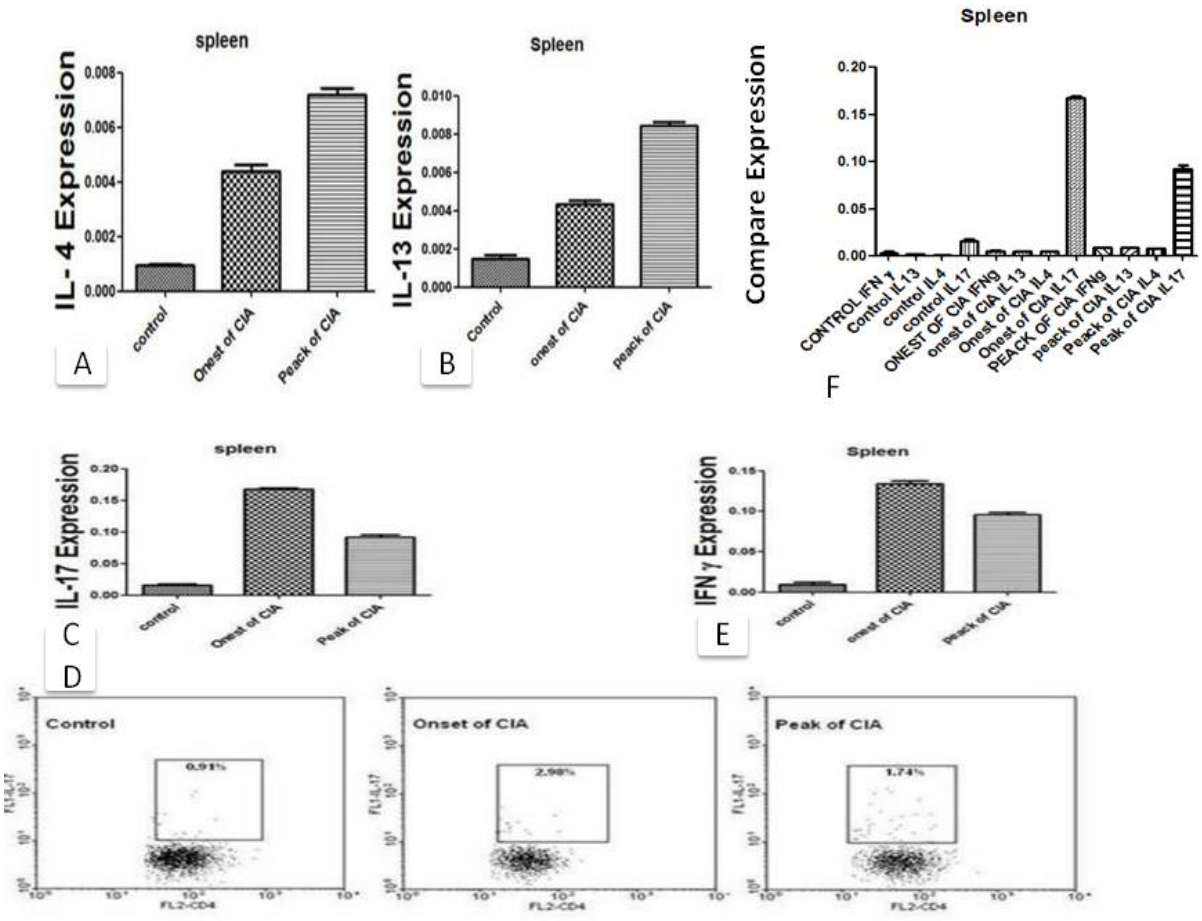

Figure 2. QPT-PCR analysis of IL-4 and IL-13 mRNA levels in spleen from DBA/1 WT normal the peak of CIA in the spleen ($p < 0.05$). **(C).** QPT-PCR analysis of IL-17 mRNA levels in spleen from DBA/1 WT normal and diseased mice (n=8 mice per group). IL-17 increased significantly at the onset of CIA in spleen ($p<0.01$). **(D).** Flowcytometry analysis of IL-17 expression in the pathogenesis of CIA. The result is the same as qRT-PCR. **(E).** QRT-PCR and flow cytometry analysis of the IFN-γ expression in the disease process showing increased IFN-γ at the onset of CIA and its decrease in the peak of CIA. **(F).** QRT-PCR analysis comparing IL-4 and IL-13 levels with IL-17 level. IL-17 level is higher than IL-4 and IL-13 levels at the onset of RA.

Involvment of IL-4 in CIA. During CIA priming, a broad range of cytokines were induced in DBA/mice and it was found that IFN-γ and IL-17 expressions were higher than that of IL-4 and IL-13. To determine the IL-4 role in inflammation, the expression of IL-4 mRNA was analyzed and the result showed that IL-4 and IL-13 gradually increased during CIA (Figures 2A and 2B).

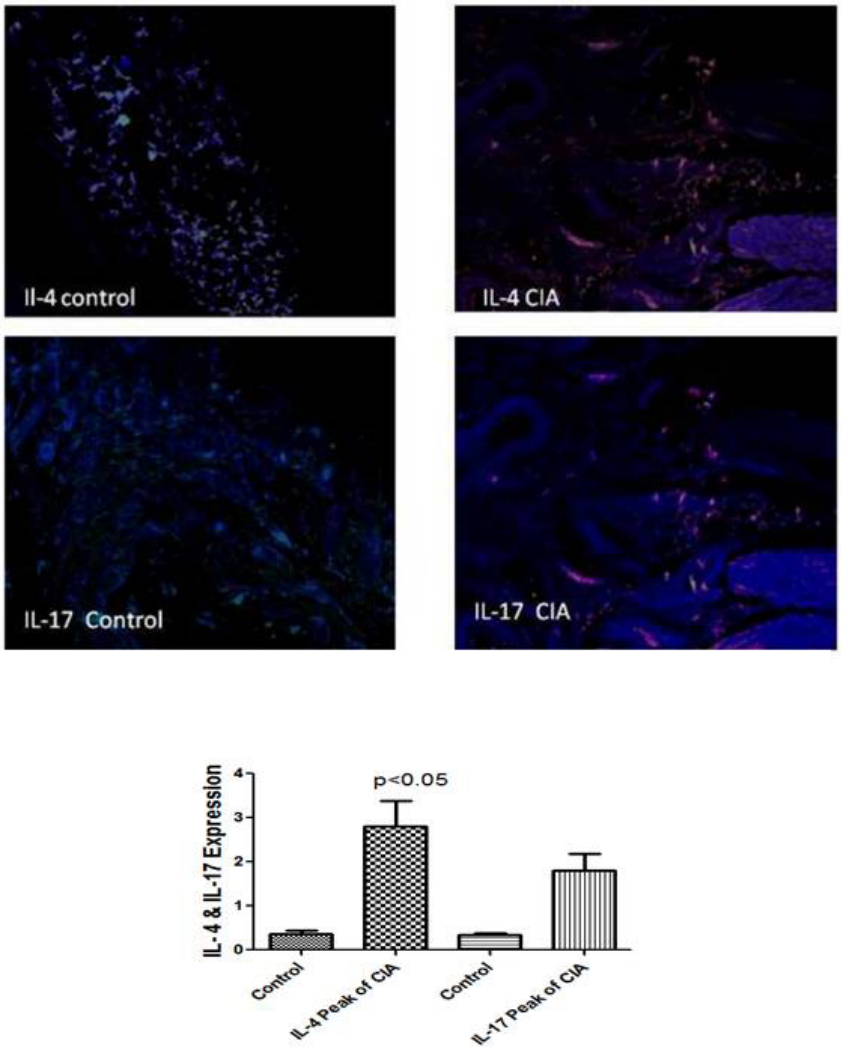

Figure 3. Immunofluorescence showing the increased expression of IL-4 in CIA group compared with the control group especially at the peak of CIA where IL-4 is higher than the early CIA. Also the result showed that when IL-4 is increased, IL-17 is decreased concluding that IL-4 can inhibit IL-17 ($p<0.05$).

Effect of IL-4 on IL-17 Level. IL-17 plays an important role in rheumatoid arthritis. The qRT-PCR and flow cytometry (Applied Biosystem, USA) results showed that in the peak of RA, IL-17 is reduced although it was highly expressed at the onset of CIA (Figure 2C and 2D). However, during inflammation, IL-4 expression was increased. Further study showed that the balance of Th1/Th2-type cytokines may have a substantial role in the regulation of an autoimmune disease. Our data also indicated that IFN-γ was always at high levels in the course of the disease (Figures 2E and 2F).
Immunofluorescence data also showed that IL-4 expression in the knee of DBA/mice at the peak of CIA was higher than IL-17 expression in that area ($p<0.05$) (Figure 3). To ascertain this point, we used anti-IL-4 antibody in the spleen cell culture; the result of which showed that before using anti-IL-4 antibody, the IL-17 expression at the peak of RA was higher and the expression of IFN-γ was also increased (the result not shown).

Expression of IL-4 Can Decrease the Proinflammatory Cytokines. IL-4 protein levels were determined in serum and spleen cell culture supernatants by ELISA (n=6 mice per group) demonstrated that IL-4 expression increased slightly in the serum ($p<0.05$) and in the spleen cell culture supernatants ($p<0.03$) at the peak of CIA.

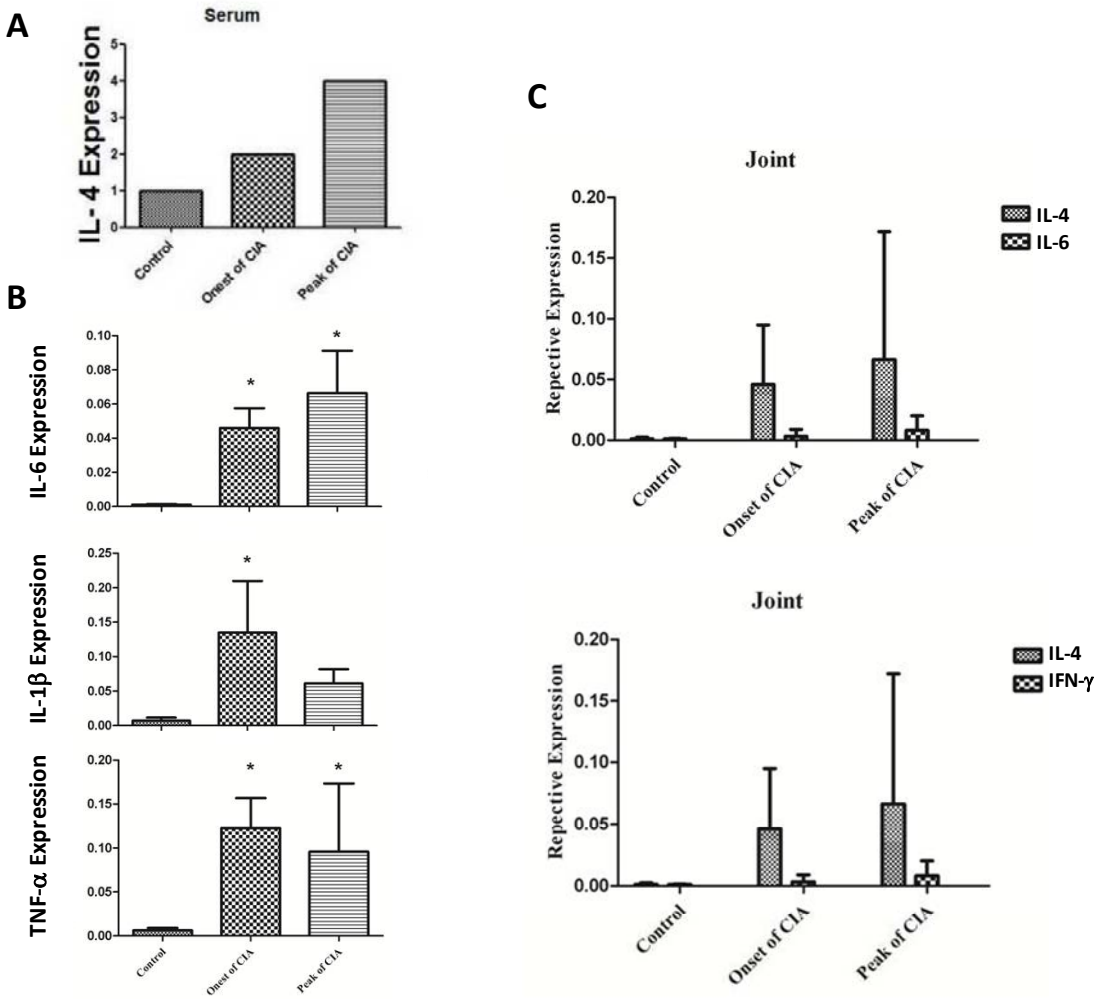

Figure 4. A: IL-4 expression in serum and spleen cell culture in DBA mice increases during the CIA ($p<0.05$) **B:** ELISA results showing that IL-1β, TNF-α and IL-6 expression in serum is higher in CIA mice ($p<0.05$) **C:** ELISA results from joint cells showing the relationship between IL-4 and IL-6 or IFN-γ. IL-4 can inhibit both IL-6 and IFN-γ expression ($p<0.05$).

Similarly, IL-4 expression showed no significant change (Figure 4A). IL-6 and IL-1β and TNF-α levels were determined in mice sera by ELISA assay (n=6). The results showed that the expression of these cytokines were increased in sera of the CIA mice ($p<0.05$) (Figure 4B). Also the results from the joint cell studies showed that IL-6 and IFN-γ decrease in the presence of IL-4 (Figure 4C).

DISCUSSION

During the pathogenesis of CIA, some pro-inflammatory cytokines accelerate the synovial pathology of RA. IL-6 and transforming growth factor (TGF)-β, IL-21 and IL-23 are important in the generation, expansion and maintenance of Th17 cells (11). All of these Th17-associated cytokines are found in RA synovial tissue. In addition, IL-17 can synergize with IL-1β, a cytokine known to play an important role in the pathogenesis of RA (12). Although there have been significant advances in understanding the development and maintenance of Th17 cells in vitro, the endogenous regulation of Th17 responses during the development of arthritis is still under investigation (13). In this study, we demonstrated the effects of IL-4 on Th1/Th2 balance following antigenic stimulation. Studies have found that IL-4 and IL-17 play opposing roles in certain diseases. $CD4^{+}CD25^{+}$ Treg cells serve an important function in the regulation of autoimmune diseases (14,15). IL-4 production is important to NK T-cells, which secrete IL-4, IL-5 and IL-13 during the immune response. Meanwhile, a histological assessment of the hind paws from arthritic DBA mice revealed that inflammatory cell infiltration was increased in DBA mice compared with the control. The qRT-PCR and flow cytometry results showed that in the peak of RA, IL-17 is reduced even though it was highly expressed at the onset of CIA (Figures 2C and 2D). Also during inflammation, IL-4 expression was increased. Several studies have shown that a balance of Th1/Th2-type cytokines may have a substantial role in the regulation of autoimmune diseases. Our data also showed that IFN-γ was always at high levels in the course of the disease (Figure 2E). Immunofluorescence data also demonstrated IL-4 and IL-17 expression from the knee of DBA/mice (Figure 3). Some cytokines such as IL-25 may play a role in macrophages caused by the secretion of several cytokines (such as IL-4) to participate in the humoral immune response (16). Results of our study suggest that disease outcome is not determined solely by the absolute level of the pathogenic cytokine, but rather by the balance between pathogenic and protective signals. One possibility is that, these signals modulate trafficking of Th17 cells to the joint, either by altering the expression of chemokines by cells of the synovium or expression of chemokine receptors by T cells. Once in the joint, Th17 cells can then induce inflammation and recruitment other inflammatory cells (17). Interestingly, after immunization, similar high levels of IL-17 were detectable in the serum of both arthritic and non-arthritic animals, but this cytokine was only found in the arthritic joints. Non-arthritic paws from arthritic mice or paws from non-arthritic mice do not have detectable IL-17. Our results implied that the balance between Th1 and Th17 cells played an important role in the disease.

In summary, these observations support the role of Th-17 cells in the pathogenesis of RA and also support the role of IL-4 in the inhibition of IL-17 which may imply that the inhibition of IL-17 can decrease the expression of IL-1β and IL-6 production which will result in the aggravation of arthritis.

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