

## Expression of IL-23 and IL-17 and effect of IL-23 on IL-17 production in ankylosing spondylitis

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**Abstract** The objective of this study was to investigate the expression of IL-23 and IL-17 and the influence of IL-23 on IL-17 production in ankylosing spondylitis (AS) patients. IL-23 and IL-17 levels in the serum and supernatants of cultured peripheral blood mononuclear cells (PBMCs) were determined by ELISA. IL-23p19 mRNA expression in PBMCs were analyzed using RT-PCR. The patients with AS at active stage showed elevated levels of IL-23 and IL-17 in the serum and supernatants of cultured PBMCs. A higher expression of IL-23p19 mRNA in PBMCs of AS patients was also observed. A significantly enhanced production of IL-17 in the supernatants of cultured PBMCs was found in the presence of recombinant IL-23 and this effect was more significant in patients with AS. The results suggest that IL-23 and IL-17 may play critical roles in the pathogenesis of AS and IL-23-stimulated production of IL-17 by PBMCs may be responsible for the development of AS.

**Keywords** Ankylosing spondylitis · IL-23 · IL-17

### Introduction

IL-23 is a heterodimeric protein that is a member of the IL-12 family of cytokines. It is composed of a unique

p19 and a common p40 subunit that is shared with IL-12 [1]. IL-23 is produced by activated monocytes, activated antigen-presenting cells including DCs and macrophages, T cells, B cells and endothelial cells [1–4]. IL-23 plays a central role in the expansion and survival of IL-17-producing Th17 cells which are responsible for many inflammatory autoimmune diseases once attributed to Th1 cells [5, 6]. Recent studies have found that IL-23 was increased in patients with psoriasis and Behcet's disease [2, 7]. The expression of IL-17 was upregulated in certain autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, psoriasis and inflammatory bowel disease [8–14]. IL-23 and IL-17 have been shown to play critical roles in the development and maintenance of autoimmune inflammation [5, 6, 15–20]. The mechanisms by which IL-23 induces autoimmunity are not completely understood. Accumulating evidences indicate that IL-17 may be one of the important effectors involved in these mechanisms.

Ankylosing spondylitis (AS) is an autoimmune disease characterized by inflammatory back pain, asymmetrical peripheral oligoarthritis (predominantly of the lower limbs), enthesitis, and specific organ involvement such as anterior uveitis, psoriasis and chronic inflammatory bowel disease. The cause of AS is unknown now. The two central features that deserve explanation are inflammation and new bone formation, especially in the spine [21]. Although previous studies suggested that IL-23 and IL-17 may play critical roles in certain autoimmune diseases, it is not clear whether they are involved in AS yet. Our study was therefore designed to examine the expression of IL-23 and IL-17 and the effect of IL-23 on the production of IL-17 by peripheral blood mononuclear cells (PBMCs) from AS patients. In this

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study, our results showed that IL-23 and IL-17 levels in the serum and supernatants of PBMCs were significantly higher in AS patients compared with normal controls. IL-23p19 mRNA expression in PBMCs was markedly up-regulated in AS patients. Recombinant (r) IL-23 could promote higher IL-17 production of PBMCs in AS patients than in normal controls. Taken together, our findings suggest that IL-23 and IL-17 may play critical roles in the development of AS.

## Materials and methods

### Patients

This study was approved by the ethics committee of the third affiliated hospital of Sun Yat-Sen University. Informed consent was obtained from all patients and control subjects. Fifty-seven patients with AS disease (52 men and 5 women) with an average age of 29.7 years and 38 healthy individuals (30 men and 8 women) with an average age of 27.7 years were included in the study. All AS patients were diagnosed according to the New York modified criteria [22]. Eighteen patients were diagnosed for the first time and all the patients' BASDAI were no <4 when taken blood samples.

### Cell isolation and culture

The PBMCs were prepared from heparinized venous blood by Ficoll–Hypaque density-gradient centrifugation. Cells were grown in RPMI1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) at an initial density of  $2 \times 10^6$  cells/ml. To study the production of IL-23, isolated PBMCs were divided into two aliquots: one was stimulated with or without *Staphylococcus aureus* Cowan I (SAC, Sigma, USA) (0.02%) for 4 h and analyzed for IL-23p19 subunit using RT-PCR, and the other was stimulated for 48 h and subsequently used for IL-23 analysis by ELISA. For determination of IL-17 production, isolated PBMCs were cultured in the presence or absence of rIL-23 (50 ng/ml) (R & D Systems, USA) for 72 h.

### ELISA for IL-23 and IL-17

The concentrations of IL-23 in the serum and supernatants were assayed using a human IL-23 ELISA kit (Bender MedSystems, Austria). The concentrations of IL-17 in the serum and the collected supernatants were measured using a human IL-17 ELISA kit (R & D Systems, USA).

### RNA preparation and RT-PCR

Total RNA was extracted from PBMCs using Trizol Reagent (Invitrogen). After precipitation with isopropanol and washing with 75% ethanol, the RNA pellets were dissolved in diethylpyrocarbonate-treated water. mRNA from 1  $\mu$ g of total cellular RNA was reversed and transcribed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) in 20  $\mu$ l final volume reactions containing oligo dT<sub>18</sub>, 5 $\times$  reaction buffer, Ribobock™ Ribonuclease, dNTP and M-Mulv. PCR amplification of cDNA aliquots was performed by adding 1.25 mM of each dNTP, 1.25 U *Taq* DNA polymerase (Takara) and 20  $\mu$ M of sense and antisense primers. The reaction was done in 25  $\mu$ l of PCR buffer (15 mM MgCl<sub>2</sub>, 500 mM KCl, 100 mM Tris–HCl, pH 8.3). The sense and antisense primers used in this experiment were: IL-23p19: sense 5'-TGT GGA GAT GGC TGT GAC-3', antisense 5'-TTG AAG CGG AGA AGG AGA-3',  $\beta$ -actin: sense 5'-GGA CTT CGA GCA AGA GAT GG-3', antisense 5'-TGT GTT GGC GTA CAG GTC TTT G-3'. The expected product lengths were 275 bp for p19 and 229 bp for  $\beta$ -actin. Reactions were processed in a DNA thermal cycler as follows: 94°C for 5 min followed by 30 cycles of: 30 s of denaturation at 94°C, 45 s of annealing at 55°C for IL-23p19 and at 58°C for  $\beta$ -actin, followed by 45 s of elongation at 72°C. Finally, the PCR products were fully extended by incubation at 72°C for 5 min. PCR products were run on a 1.8% agarose gel and stained with ethidium bromide. The level of mRNA expression was presented as a ratio of IL23p19 PCR product over  $\beta$ -actin product.

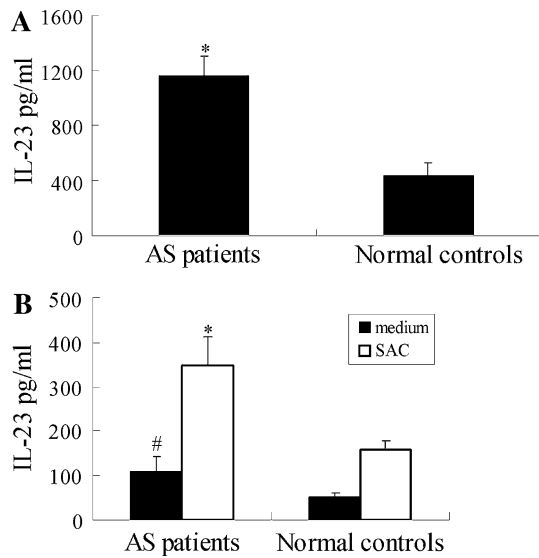
### Statistical analysis

Data were expressed as mean  $\pm$  SD. Statistical analysis was performed using Student *t* test. A level of  $P < 0.05$  was considered to be statistically significant.

## Results

### Levels of IL-23 in the serum and supernatants of cultured PBMCs of AS patients and normal control subjects

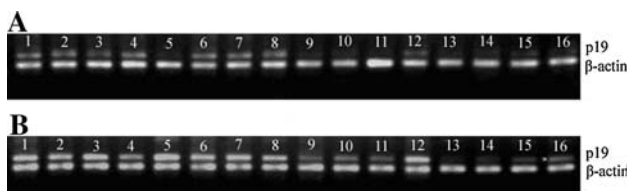
Serum IL-23 levels were significantly higher in AS patients ( $n = 39$ ) than those in normal control subjects ( $n = 20$ ;  $P < 0.001$ ; Fig. 1a). The expression of IL-23 in the supernatants of PBMCs being cultured 48 h in AS patients ( $n = 8$ ) were significantly higher than that in normal control subjects ( $n = 8$ ;  $P = 0.002$ ) as well. IL-23 production was significantly increased in the presence of SAC stimulation, with a higher upregulation in AS patients than in normal control subjects ( $P < 0.001$ ; Fig. 1b).



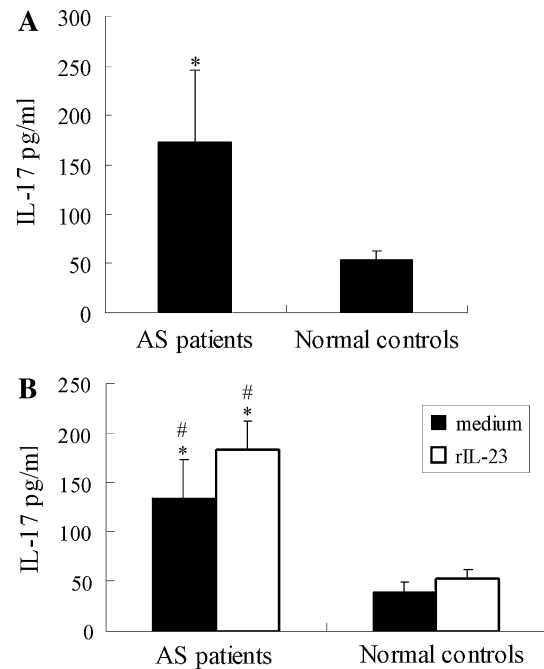
**Fig. 1** IL-23 levels in the serum and supernatants of cultured PBMCs. **a** IL-23 levels in the serum of AS patients ( $n = 39$ ) and normal control subjects ( $n = 20$ ) measured by ELISA. **b** IL-23 levels in the supernatants of cultured PBMCs from AS patients ( $n = 8$ ) and normal control subjects ( $n = 8$ ) determined by ELISA. Separated PBMCs were cultured with or without SAC (0.02%) for 48 h. Data are represented as mean  $\pm$  SD. \* $P < 0.001$  compared with normal control subjects. # $P < 0.01$  compared with normal control subjects

Expression of IL-23p19 mRNA in PBMCs of AS patients and normal control subjects

We investigated IL-23p19 mRNA expression of PBMCs in AS patients and normal control subjects. The expression of IL-23p19 mRNA was normalized by respective  $\beta$ -actin mRNA. The average intensity ratio of IL-23p19 PCR product/ $\beta$ -actin product ( $0.466 \pm 0.152$ ) in AS patients ( $n = 8$ ) was significantly higher than that in normal control subjects ( $0.226 \pm 0.048$ ;  $n = 8$ ;  $P = 0.003$ ; Fig. 2a). Moreover, IL-23p19 mRNA was also markedly higher in AS patients ( $0.972 \pm 0.116$ ) than that in normal control subjects ( $0.480 \pm 0.269$ ;  $P < 0.001$ ) after stimulation with SAC (Fig. 2b). Data were represented as mean  $\pm$  SD.



**Fig. 2** RT-PCR analysis of IL-23p19 in PBMCs from AS patients ( $n = 8$ ) and normal control subjects ( $n = 8$ ). **a** RNA was extracted from PBMCs of AS patients (lanes 1–8) and normal control subjects (lanes 9–16). **b** PBMCs were stimulated by SAC for 4 h

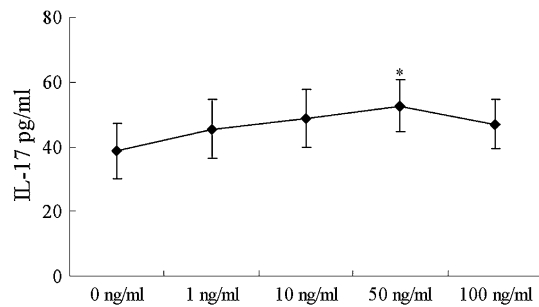


**Fig. 3** IL-17 levels in the serum and supernatants of cultured PBMCs. **a** IL-17 levels in the serum of AS patients ( $n = 39$ ) and normal control subjects ( $n = 20$ ) measured by ELISA. **b** IL-17 production by cultured PBMCs from AS patients ( $n = 10$ ) and normal control subjects ( $n = 10$ ). Cells were cultured in the presence or absence of rIL-23 for 72 h. \* $P < 0.001$  compared with normal control subjects; # $P < 0.01$  compared with medium. Data are represented as mean  $\pm$  SD. \* $P < 0.001$  compared with normal control subjects, # $P < 0.01$  compared with medium

Concentrations of IL-17 in the serum and supernatants of cultured PBMCs of AS patients and normal control subjects and effect of IL-23 on the production of IL-17

IL-17 levels in serum were significantly higher in AS patients ( $n = 39$ ) than those in normal control subjects ( $n = 20$ ;  $P < 0.001$ ; Fig. 3a). Although very low levels of IL-17 were detected in the supernatants of PBMCs being cultured 72 h, a significantly higher expression was noted in AS patients ( $n = 10$ ) than that in normal control subjects ( $n = 10$ ;  $P < 0.001$ ; Fig. 3b).

To examine the influence of rIL-23 on the production of IL-17, PBMCs separated from five healthy individuals were cultured in the presence of rIL-23 (0, 1, 10, 50, 100 ng/ml) for 72 h. The results showed that the production of IL-17 was augmented significantly at dose of 50 ng/ml of rIL-23 ( $P = 0.028$ ; Fig. 4) and this concentration was therefore used for subsequent experiments. The similarly stimulatory effect of IL-23 on IL-17 production was also seen in stimulated PBMCs of AS patients ( $P = 0.005$ ). This effect was more pronounced in AS patients than in normal control subjects ( $P < 0.001$ ; Fig. 3b).



**Fig. 4** Recombinant IL-23 induces IL-17 production by PBMCs from normal control subjects ( $n = 5$ ). PBMCs were cultured in the presence or absence of rIL-23 (0, 1, 10, 50, 100 ng/ml) for 72 h. The concentrations of IL-17 in the supernatants of cultured PBMCs were measured by ELISA. Data are expressed as mean  $\pm$  SD. \* $P < 0.05$  compared with 0 ng/ml

## Discussion

In the present study, we found elevated levels of IL-23 and IL-17 in the serum and supernatants of cultured PBMCs of AS patients. Moreover, an increased expression of IL-23p19 mRNA was also found in PBMCs of AS patients. Furthermore, we observed that IL-23 could promote IL-17 production by PBMCs of AS patients and normal control subjects. IL-23 stimulated a considerably higher production of IL-17 by PBMCs of AS patients than of normal control subjects. These results suggest that IL-23 and IL-17 may be involved in the pathogenesis of AS and IL-23 may play a unique role possibly through promoting the secretion of IL-17.

To study whether IL-23 is involved in the development of AS patients, we first assayed its levels in the serum of AS patients and normal control subjects. We found that serum IL-23 levels were markedly increased in AS patients. Because IL-23 is primarily produced by activated antigen-presenting cells that are present in PBMCs, we further assayed the production of IL-23 by PBMCs [23]. It has been reported that IL-23 is composed of a unique p19 and a common p40 subunit shared with IL-12 [1]. Therefore, we only examined the IL-23p19 mRNA expression in PBMCs by RT-PCR. The results showed an increased expression of IL-23p19 mRNA in PBMCs of AS patients than that of normal control subjects. We also observed a similar result for the IL-23 level in the supernatants of unstimulated cultured PBMCs. As previous study has shown that SAC was a potent stimulator for IL-23 secretion [23], we further tested the production of IL-23 by PBMCs stimulated by SAC. The results showed that both IL-23 levels in the supernatants of cultured PBMCs and IL-23p19 mRNA in PBMCs were markedly increased in AS patients and normal control subjects following stimulation with SAC. Moreover, the SAC-induced upregulation of IL-23 and IL-23p19 mRNA

was significantly increased in AS patients. Our studies revealed a strong association between elevated levels of IL-23 and AS. These results suggest that IL-23 may be actively involved in the pathogenesis of AS.

IL-17 has been claimed to function as an effector cytokine primarily induced by IL-23 [16, 24, 25]. To investigate whether the upregulated IL-23 in AS patients promotes the disease via induction of IL-17, we measured its production in patients and controls. An elevated level of IL-17 in the serum was observed in AS patients than in normal control subjects. Furthermore, we assayed IL-17 production by PBMCs from AS patients and normal control subjects. The results showed a significantly increased production of IL-17 in the supernatants of PBMCs in AS patients compared with normal control subjects. Because CD4<sup>+</sup> T cells play an essential role in the development of autoimmune diseases and have been indicated highly pathogenic on some autoimmune disease models, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis, it will be more instructive to address IL-17 production by CD4<sup>+</sup> T cells in AS patients [24–27].

As IL-23 and IL-17 were coincidentally increased in AS patients and IL-17 can be induced by IL-23, we further examined whether IL-23 could enhance the production of IL-17 in AS. Our findings showed that IL-23 could induce PBMCs to secrete IL-17 in AS patients and normal control subjects. More importantly, IL-23-induced IL-17 production by PBMCs was markedly higher in AS patients than in normal control subjects. These results revealed that IL-23 could induce IL-17 production and that the PBMCs of AS patients most highly responded to the IL-23 stimulation. This study suggest that the up-regulated IL-23 in AS patients may sensitize certain effector cells in PBMCs to produce IL-17 which in turn participates in the development of AS.

In conclusion, our study revealed that the levels of IL-23 and IL-17 in serum and supernatants of PBMCs and the expression of IL-23p19 mRNA in PBMCs are all significantly increased in AS patients. Furthermore, IL-23 enhances the production of IL-17 by PBMCs of AS patients and normal control subjects. These results suggest that upregulated IL-23 and IL-17 production may be responsible for the development of AS. As many other cytokines could influence the production of IL-17, it is necessary to examine the role of other cytokines on the production of IL-17 in AS patients. It is also important to explore the mechanisms of IL-23-induced IL-17 production in AS. All in all, an in-depth understanding of the IL-23/IL-17 pathway may contribute to the study on the pathogenesis and new therapeutic target of AS.

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