Upregulated IL-23 and IL-17 in Behçet Patients with Active Uveitis

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PURPOSE. Behçet disease (BD) is a systemic inflammatory disease presumably caused by an autoimmune response. The interleukin (IL)-23/IL-17 pathway has been demonstrated to be involved in the development and maintenance of certain inflammatory diseases. This study was designed to investigate the role of IL-23 and IL-17 in BD.

METHODS. IL-23p19 mRNA in peripheral blood mononuclear cells (PBMCs) was examined using RT-PCR. The levels of IL-23, IL-17, and IFN-γ in sera or PBMCs were detected by ELISA. Flow cytometry was used to evaluate the frequencies of IL-17–producing and IFN-γ–producing T cells and the expression of CD45RO.

RESULTS. Results showed that the expression of IL-23p19 mRNA, IL-23, IL-17, and IFN-γ was markedly elevated in BD patients with active uveitis. The frequencies of IL-17–producing and IFN-γ–producing T cells from PBMCs were significantly upregulated in BD patients with active uveitis. The increased IL-17 (3.10% ± 0.53%) in BD patients with active uveitis was primarily produced by CD45RO+ memory T cells. Recombinant (r) IL-23 could upregulate IL-17 production by polyclonally stimulated PBMCs, whereas interferon (IFN)-γ downregulated IL-17 production.

CONCLUSIONS. These findings reveal that the levels of IL-23, IL-17, and IFN-γ are elevated in BD patients with active uveitis, and they suggest that the IL-23/IL-17 pathway together with IFN-γ is associated with the active intraocular inflammation in BD patients. (Invest Ophthalmol Vis Sci. 2008;49:3058-3064) DOI:10.1167/iovs.07-1390

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Behçet disease (BD) is a chronic systemic inflammatory disease characterized by recurrent uveitis, oral aphthae, genital ulcers, and skin lesions. Although the pathogenesis of BD is still unclear, several reports have suggested that autoreactivity may play a crucial role.1,2 Previous studies suggested that BD is predominated by a Th1-type immune response. Increased Th1-associated cytokines such as interferon (IFN)-γ, interleukin (IL)-12, and tumor necrosis factor (TNF)-α have been documented in BD patients.3,4 However, treatment with IFN-α and anti-TNF-α could only partially prevent the progression of BD.5 Therefore, other factors seem to be involved in the development of BD.

Recent studies have shown that IL-17 is an important proinflammatory cytokine and is upregulated in certain inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease (IBD), multiple sclerosis, uveitis including BD, and Vogt-Koyanagi-Harada disease.6–10 IL-23, a novel member of the IL-12 family, has been shown to be necessary for the development and maintenance of certain inflammatory autoimmune disease models such as IBD, autoimmune encephalomyelitis, collagen-induced arthritis, and experimental autoimmune uveitis.11–15 It is also demonstrated that IL-23 is able to amplify and stabilize Th17 cells in disease models and humans.11–16 These results suggest that the IL-23/IL-17 pathway may play a role in these diseases. It is not yet clear whether the IL-23/IL-17 pathway is involved in the development of BD, though an increased level of IL-17 has been found in the sera of patients with this disease.4 Therefore, the study was designed to investigate the association of IL-23 and IL-17 with the intraocular inflammation seen in BD patients.

The present study showed that IL-23 in the sera and IL-23p19 mRNA and IL-23 protein levels in PBMCs were upregulated in BD patients with active uveitis. The production of IL-17 and IFN-γ by polyclonally stimulated PBMCs and activated T cells was significantly elevated in BD patients with active uveitis. These data suggest that upregulated IL-23 and IL-17 production is associated with active intraocular inflammation in BD patients.

PATIENTS AND METHODS

Patients

Twenty-three BD patients (18 men, 5 women) referred to us from May 2005 to September 2007, with an average age of 36.3 years, and 19 healthy persons (14 men, 5 women), with an average age of 37 years, were included in this study. The diagnosis of BD disease was based on the diagnostic criteria designed by the International Study Group for BD disease.17,18 Fourteen patients showed active recurrent intraocular inflammation. These patients had received only prednisone at a low dose (<20 mg/d), but no other immunosuppressive agents, for the treatment of uveitis for more than 2 months before blood sample collection. Blood samples were collected from all these patients with active uveitis and from healthy controls. These patients showed active inflammation.
recurrent intraocular inflammation evidenced by dust keratic precipitates (100%), flare and cells in the anterior chamber (100%) and vitreous cells (46.7%), and retinal vasculitis observed clinically or disclosed by fluorescein angiography (100%). The extraocular manifestations were recurrent oral aphthous lesions (100%), multifocal skin lesions (66.7%), recurrent genital ulcers (44.4%), and arthritis (33.3%). Nine patients showed inactive intraocular inflammation after treatment with prednisone combined with cyclosporin A for more than 3 months. Blood samples were obtained from these patients without active uveitis at least 2 months after the termination of all medications. The experimental design is described in detail. All procedures followed the tenets of the Declaration of Helsinki, and informed consent was obtained from all patients and healthy controls.

**Analysis of IL-23p19 mRNA Using RT-PCR**

RT-PCR analysis was performed using the reagents and procedures described previously.10

**Measurement of Cytokines by ELISA**

PBMCs were stimulated with or without *Staphylococcus aureus* Cowan 1 (SAC; 0.02%; Sigma-Aldrich, St. Louis, MO) for 72 hours at a density of 2 × 10^6^ cells/mL. IL-23 concentrations were measured by human IL-23 ELISA kit (R&D Systems, Minneapolis, MN) with a detection limit of 15 pg/mL.

For determination of IL-17 and IFN-γ production, PBMCs were stimulated with or without anti-CD3 (5 μg/mL; eBioscience, San Diego, CA) and anti-CD28 antibodies (1 μg/mL; eBioscience) in the presence or absence of rIL-23 (50 ng/mL), rIL-12 (1 ng/mL), or anti-IFN-γ (10 μg/mL; R&D Systems) for 72 hours. The reagents and procedures were performed as described previously.10 For determination of the influence of IL-12 or IFN-γ on IL-17 production, PBMCs isolated from five healthy controls were stimulated with anti-CD3 and anti-CD28 antibodies in the presence or absence of anti-IFN-γ (10 μg/mL) plus rIL-12 (1 ng/mL) or rIL-23 (50 ng/mL) or in the presence of rIFN-γ (100 ng/mL; eBioscience) plus anti-IL-12p70 (10 μg/mL; R&D Systems) for 72 hours. The supernatants were used for measurement of IL-17 using ELISA.

**Intracellular Cytokine Staining**

In total, 2 × 10^6^ cells/mL PBMCs were stimulated with 20 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin (Sigma) for 24 hours. During the final 4 hours, 10 μg/mL Brefeldin A (Sigma) was added to the cultured PBMCs. The stimulated PBMCs were washed in PBS and incubated with phycoerythrin (PE)-cy7-labeled anti-CD8, allophycocyanin-labeled anti-CD45RO, FITC-labeled anti-CD69, or matched isotype (eBioscience; San Diego) for 30 minutes in the dark at 4°C. These PBMCs were fixed in 4% formaldehyde, permeabilized with 0.1% saponin (Sigma), and stained with PerCP-labeled anti-CD3 (BD PharMingen, San Diego, CA), PE-labeled anti-IL-17, PE-labeled anti-IFN-γ (eBioscience), or matched isotype control mAb. Cells (1 × 10^6^) were analyzed using a FACSCalibur and CellQuest software (BD PharMingen).

**Statistical Analysis**

Data are expressed as mean ± SD. Statistical analysis was performed using Student’s *t*-test and one-way ANOVA. *P* < 0.05 was considered statistically significant.

**RESULTS**

**Increased Expression of IL-23p19 mRNA in PBMCs from BD Patients with Active Uveitis**

Sequenced PCR products of IL-23p19 displayed 99.6% homology with the known IL-23p19 sequence. The intensity of the IL-23p19 mRNA transcript products was normalized by respective β-actin mRNA transcript products. Significantly increased expression of IL-23p19 mRNA was observed in BD patients with active uveitis (0.27 ± 0.04) without stimulation compared with that in BD patients without active uveitis (0.08 ± 0.03; *P* < 0.001; Fig. 1A). We further investigated the expression of IL-23p19 mRNA in PBMCs after stimulation with SAC. The results showed that IL-23p19 mRNA expression was significantly increased on SAC stimulation in patients and in controls. Furthermore, SAC-stimulated IL-23p19 mRNA was also significantly higher in BD patients with active uveitis (1.04 ± 0.37) than in BD patients without active uveitis (0.21 ± 0.08; *P* < 0.001) and healthy controls (0.16 ± 0.04; *P* < 0.001; Fig. 1B).

**Elevated Levels of IL-23 in the Sera and the Supernatants of Cultured PBMCs from BD Patients with Active Uveitis**

IL-23 levels in the sera were significantly upregulated in BD patients with active uveitis compared with that in BD patients without active uveitis (*P* < 0.001) and healthy controls (*P* < 0.001) (Fig. 2A). The expression of IL-23 in the supernatants of cultured PBMCs was significantly higher in BD patients with active uveitis than in BD patients without active uveitis (*P* < 0.001) and healthy controls (*P* = 0.004). On SAC stimulation, IL-23 production was obviously elevated in all the tested samples. Moreover, its elevation was significantly higher in BD patients with active uveitis than in BD patients without active uveitis (*P* < 0.001) and healthy controls (*P* = 0.002; Figure 2B).

**Increased Expression of IL-17 and IFN-γ in the Supernatants of Stimulated PBMCs and Activated T Cells from BD Patients with Active Uveitis**

The level of IL-17 was undetectable in the sera of all patients and controls. The production of IL-17 was strikingly increased in PBMCs from BD patients with active uveitis compared with that in BD patients without active uveitis and in healthy controls (Fig. 3A). Moreover, its elevation was significantly higher in BD patients with active uveitis than in BD patients without active uveitis (0.08 ± 0.001; Fig. 3A). IFN-γ was also undetectable in the sera or the supernatants of unstimulated PBMCs from BD patients and healthy controls. On stimulation with anti-CD3 and anti-CD28 antibodies, the production of IFN-γ by PBMCs was markedly increased in patients and in healthy controls. Such increases were significa-

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**Figure 1.** IL-23p19 mRNA in PBMCs from BD patients with (*n* = 8) or without (*n* = 6) active uveitis and healthy controls (*n* = 6). (A) IL-23p19 mRNA in unstimulated PBMCs. (B) IL-23p19 mRNA in SAC-stimulated PBMCs. IL-23p19 mRNA expression was significantly higher in BD patients with active uveitis (*lanes 1–3*) than in BD patients without active uveitis (*lanes 4–6*) and healthy control (*lanes 7–9*).
patients and healthy controls than was the percentage of IFN-γ-producing CD4+ T cells (P = 0.021; Figs. 4A, 4B). In view of the fact that CD4+ and CD8+ T cells are functionally and phenotypically divided into naïve and memory T cells according to differential expression of the CD45 isotype on their surfaces,19 we further investigated the expression of CD45RO on these IL-17-producing T cells. The results showed that IL-17 was principally expressed by CD4+CD45RO+ and CD8+CD45RO+ memory T cells in BD patients and healthy controls after stimulation with PMA and ionomycin. The frequencies of IL-17-producing CD4+CD45RO+ memory T cells were significantly higher in BD patients with active uveitis than in BD patients without active uveitis (P < 0.001) and healthy controls (P = 0.015; Fig. 5).

Effect of IL-23 and IL-12 on the Production of IL-17 and IFN-γ

To investigate the effect of IL-23 and IL-12 on IL-17 and IFN-γ, we detected IL-17 and IFN-γ production in the presence of rIL-23 and rIL-12. The results showed that rIL-23 could significantly promote the production of IL-17 by polyclonally stimulated PBMCs from BD patients with (P = 0.0008) and without (P = 0.009) active uveitis and healthy controls (P < 0.001). Moreover, the elevation was significantly higher in BD patients with active uveitis than in BD patients without active uveitis (P < 0.001) and healthy controls (P < 0.001). On the contrary, rIL-12 significantly inhibited IL-17 production by polyclonally stimulated PBMCs.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932952/)  
**Figure 2.** IL-23 in sera and PBMCs of BD patients with (n = 8) or without (n = 6) active uveitis and healthy controls (n = 6). (A) IL-23 in serum. (B) IL-23 production by PBMCs after stimulation with or without SAC. IL-23 in sera and PBMCs were significantly higher in BD patients with active uveitis than in BD patients without active uveitis and healthy controls. Data are expressed as mean ± SD.

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932952/)  
**Figure 3.** IL-17 and IFN-γ production by PBMCs from BD patients with (n = 8) or without (n = 6) active uveitis and healthy controls (n = 6). (A) The production of IL-17 by polyclonally stimulated PBMCs in the presence or absence of rIL-23 (50 ng/mL) or rIL-12 (1 ng/mL). (B) The production of IFN-γ by stimulated PBMCs in the presence or absence of rIL-23 or rIL-12. The production of IL-17 and IFN-γ was significantly higher in BD patients with active uveitis than in BD patients without active uveitis and healthy controls. Data are expressed as mean ± SD.
The upregulation of IFN-γ/H9253 (10 ng/ml) for 24 hours. Cells isolated PBMCs were stimulated with PMA/ionomycin for 24 hours. Cells (1 x 10⁶) were used for FACS analysis. (A) Representative dot plots from five independent experiments illustrated the higher frequencies of IL-17– and IFN-γ–producing CD4+ and CD8+ T cells in BD patients with active uveitis compared with those in BD patients without active uveitis and healthy controls. We first gated CD3+ T cells and then gated CD3+CD8+ T cells and CD3+CD8+ T cells. Quadrant statistics represent the frequencies of IL-17- and IFN-γ-producing cells within gated CD3+CD8+ T cells and CD3+CD8+ T cells. (B) Percentages of IL-17–producing and IFN-γ–producing CD4+ and CD8+ T cells were significantly higher in BD patients with active uveitis than in BD patients without active uveitis and healthy controls. Data are expressed as mean ± SD.

![Diagram showing percentages of IL-17 and IFN-γ production](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932952/)

**FIGURE 4.** The percentages of IL-17–producing and IFN-γ–producing T cells from BD patients with (n = 11) or without (n = 8) active uveitis and healthy controls (n = 8). Isolated PBMCs were stimulated with PMA/ionomycin for 24 hours. Cells (1 x 10⁶) were used for FACS analysis. (A) Representative dot plots from five independent experiments illustrated the higher frequencies of IL-17– and IFN-γ–producing CD4+ and CD8+ T cells in BD patients with active uveitis compared with those in BD patients without active uveitis and healthy controls. We first gated CD3+ T cells and then gated CD3+CD8+ T cells and CD3+CD8+ T cells. Quadrant statistics represent the frequencies of IL-17- and IFN-γ-producing cells within gated CD3+CD8+ T cells and CD3+CD8+ T cells. (B) Percentages of IL-17–producing and IFN-γ–producing CD4+ and CD8+ T cells were significantly higher in BD patients with active uveitis than in BD patients without active uveitis and healthy controls. Data are expressed as mean ± SD.

from BD patients with (P = 0.041) or without (P = 0.003) active uveitis and healthy controls (P = 0.018; Fig. 3A).

rIL-23 and rIL-12 could significantly increase the production of IFN-γ by polyclonally stimulated PBMCs in BD patients with (P = 0.011 and P < 0.001, respectively) or without active uveitis (P = 0.012 and P < 0.001, respectively) and healthy controls (P = 0.008 and P < 0.001, respectively). Furthermore, the upregulation of IFN-γ by rIL-12 was significantly higher than that by rIL-23 in patients with (P < 0.001) and without (P = 0.008) active uveitis and healthy controls (P < 0.001) (Fig. 3B).

**Influence of IFN-γ on IL-17 Production**

It has been demonstrated that IFN-γ could suppress the production of IL-17 in humans and mice.10,20,21 Our further goal was to examine whether IFN-γ had the regulatory effect on IL-17 production in BD patients. The results showed that, on neutralization with anti–IFN-γ antibodies, IL-17 production was significantly increased in BD patients (P < 0.001) and healthy controls (P = 0.004; Figs. 6A, 6B). Furthermore, this increase in BD patients with active uveitis was significantly higher than it was in healthy controls.

Our results showed that rIL-12 induced a large amount of IFN-γ production and suppressed IL-17 production. IFN-γ could markedly suppress the production of IL-17. We further investigated whether IL-12 exerted its inhibitory effect on IL-17 through IFN-γ. The results showed that rIL-12 could significantly inhibit IL-17 production when anti–IL-12p70 was used in this culture (P = 0.009). rIL-12 did not suppress the production of IL-17 after IFN-γ was neutralized, demonstrating that the
suppressive effect of IL-12 on IL-17 is mediated by IFN-γ. Furthermore, our study showed that a larger amount of IL-17 was produced when rIL-23 plus anti–IFN-γ was used in this culture (Fig. 6C).

DISCUSSION
The present study showed that IL-23p19 mRNA in PBMCs, IL-23 in serum and supernatants of PBMCs, and IL-17 and IFN-γ production in supernatants of PBMCs were all markedly increased in BD patients with active uveitis. Significantly upregulated IL-17– and IFN-γ–producing T cells were also observed in BD patients with active uveitis. IL-17 was mainly expressed by CD45RO memory T cells. It was also demonstrated that IL-23 could promote IL-17 production, whereas IFN-γ downregulated IL-17 production. All these findings suggest that IL-23 and IL-17 are associated with active intraocular inflammation in BD patients.

IL-23 has been demonstrated to play a pivotal role in some inflammatory autoimmune disease models and to be associated with active BD patients with active uveitis. Increased IL-17 production in BD patients with active uveitis was also observed in BD patients with active uveitis. IL-17 was mainly expressed by CD45RO memory T cells. It was also demonstrated that IL-23 could promote IL-17 production, whereas IFN-γ downregulated IL-17 production. All these findings suggest that IL-23 and IL-17 are associated with active intraocular inflammation in BD patients.

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with certain human diseases.11-15,22,23 Accordingly, the role of IL-23 in the pathogenesis of BD was investigated in this study. First, our study showed that IL-23 was upregulated in the sera of BD patients with active uveitis compared with BD patients without active uveitis and controls. Second, IL-23p19 mRNA and IL-23 protein in PBMCs were augmented in BD patients with active uveitis. Third, our study revealed that SAC-stimulated PBMCs produced heightened IL-23p19 mRNA and IL-23 in BD patients with active uveitis. All these results suggest that upregulated IL-23 is associated with the active intraocular inflammation seen in BD.

Given that IL-17 is one of primary effectors involved in the mechanism of IL-2312,24 and that an increased expression of IL-23 in BD patients with active uveitis is observed in our experiments, we tested the expression of IL-17 in BD patients. Our results showed that IL-17 production by polyclonally stimulated PBMCs and activated T cells was markedly elevated in BD patients with active uveitis compared with that in patients without active uveitis and in controls. Moreover, our study revealed that most IL-17–producing T cells were CD4+CD45RO+ and CD8+CD45RO+; the former were predominant in patients and healthy controls. These results are consistent with those reported by Shin et al.19 In their study, IL-17 mRNA is demonstrated to be primarily expressed in CD4+CD45RO+ and CD8+CD45RO+ memory T cells from humans after polyclonal stimulation. Given that human CD45RO+ T cells are defined as antigen-experienced memory T cells,9,20 it is reasonable to presume that IL-17 is mainly produced by CD4+CD45RO+ memory T cells. More important, our results showed that the frequencies of IL-17–producing CD4+CD45RO+ and CD8+CD45RO+ T cells were higher in BD patients with active uveitis than in BD patients without active uveitis and healthy controls. In view of the high pathogenicity and the crucial effect of IL-17–producing T cells in the development and maintenance of certain autoimmune diseases,9-11 it is likely that a large amount of IL-17–producing CD4+CD45RO+ memory T cells correlates with the active intraocular inflammation in BD patients.

Because the upregulation of IL-23 and IL-17 in BD patients with active uveitis was observed in our study, we further tested the influence of IL-23 on IL-17 production. Our results showed that rIL-23 could promote the production of IL-17 by polyclonally stimulated PBMCs from BD patients and healthy controls. Moreover, rIL-23 promoted higher IL-17 in BD patients with active uveitis than in patients without active uveitis and healthy controls. These results suggested that upregulated IL-23 in BD patients with active uveitis may exert its role by promoting IL-17 production. The upregulated IL-23 and IL-17 production in BD patients with active uveitis after nonspecific stimulation in this study is consistent with the results in VKH patients with active uveitis and patients with psoriasis.10,23 Upregulated IL-23 or IL-17 has also been observed in patients with IBD22 or scleritis and uveitis,9,23 respectively. It is well known that these human autoimmune diseases display different clinical manifestations and pathologic features and that different autoantigens are involved in them. It is likely that IL-23/IL-17 may be a common pathway for these autoimmune diseases. Targeted manipulation of the IL-23/IL-17 pathway may provide insight into a new strategy for these diseases. However, it must be pointed out that upregulated IL-23 and IL-17 production in BD patients with active uveitis was found with nonspecific stimulation. Studies have shown that autoimmune responses to a number of antigens, such as S-antigen and interphotoreceptor retinoid-binding protein, are involved in the development of this disease.1,2,26 A recent study by us (not yet published) did not show any increased production of IL-17 by PBMCs from BD patients or healthy controls on stimulation with S-Ag peptides (data not shown). Nevertheless, this result does not exclude the possibility that another antigen-driven IL-23/IL-17 pathway is involved in this disease. More studies are needed to address this problem.

Because IFN-γ was considered an important mediator involved in the development of BD,3,4 a study was also performed to detect the expression of this cytokine, and importantly, to evaluate the influence of IL-23 on its production. Our results showed a significantly upregulated expression of IFN-γ in BD patients with active uveitis, confirming previous reports. Interestingly, our results also showed that IL-23 could significantly promote IFN-γ production. These results are consistent with those observed by us in Vogt-Koyanagi-Harada disease10 and with those reported by Hoeve et al.20 and suggest that IL-23 may also exert its function through induction of IFN-γ. Given that IL-12 is classically considered an inducer of IFN-γ production,27 we also tested its effects on this cytokine. The study revealed a similar result. It was also found that IL-12 and IFN-γ could inhibit the expression of IL-17. Because IFN-γ is the downstream cytokine of IL-12, we tested whether IL-12 inhibited IL-17 production directly or by the induction of IFN-γ. Our study showed that IL-12 exerted its inhibitory effect on IL-17 through IFN-γ. This finding is similar with that reported by Harrington et al.28 All these results suggest that a negative modulatory mechanism by IFN-γ is present in BD patients and that this mechanism may contribute, to a certain extent, to the outcome of the inflammation seen in these patients.

In conclusion, our study showed elevated production of IL-23, IL-17, and IFN-γ by PBMCs and increased frequencies of IL-17–producing and IFN-γ–producing T cells in BD patients with active uveitis. It also showed that IL-17 was principally produced by CD45RO+ memory T cells. All these results suggest that the IL-23/IL-17 pathway is associated with active uveitis in BD patients and that this pathway may be involved in the pathogenesis of BD. Studies on the manipulation of this pathway may warrant its role and also provide a strategy for the treatment of this disease.

References