Immune Responses to Interphotoreceptor Retinoid-Binding Protein and S-Antigen in Behçet’s Patients with Uveitis

Masaru Takeuchi, Yoshibiko Usui, Yoko Okunuki, Lina Zhang, Juan Ma, Naoyuki Yamakawa, Takaaki Hattori, Takeshi Kezuka, Jun-ichi Sakai, and Hiroshi Goto

PURPOSE. Immune responses to retina-specific autoantigens, including S antigen (S-Ag) and interphotoreceptor retinoid binding protein (IRBP), have been suggested to be involved in the pathogenesis of human uveitis, including Behçet’s disease (BD). In this study, the authors examined whether immune responses to IRBP and S-Ag in BD patients can be characterized by cytokine production profiles.

METHODS. Peripheral blood mononuclear cells (PBMCs) were collected from BD patients with uveitis and healthy controls, and each sample was cultured with IRBP, S-Ag, or purified protein derivative (PPD). At the end of culture, IL-2, IL-4, IL-6, IL-10, IL-17, INF-γ, and TNF-α concentrations in supernatants were measured.

RESULTS. PBMCs from BD patients and healthy controls produced IL-6, IL-10, IL-17, INF-γ, and TNF-α on stimulation with IRBP or S-Ag, as well as PPD stimulation, immunity against which was acquired by Bacille Calmette-Guérin immunization. IL-17 and INF-γ production was significantly higher when PBMCs were stimulated with IRBP than with S-Ag, whereas the reverse was observed for IL-6 production. IRBP-stimulated IL-6, INF-γ, and IL-17 production was higher in BD patients than in healthy controls, though IL-10 production was not different between them. In particular, IRBP-stimulated INF-γ production was significantly higher in BD patients with active uveitis than in BD patients with uveitis in remission.

CONCLUSIONS. Immune responses to both IRBP and S-Ag were observed even in PBMCs of healthy controls. However, the present results suggested that retinal autoantigen-stimulated IL-6, IL-17, and especially INF-γ production would be involved in the development of uveitis in BD. (Invest Ophthalmol Vis Sci. 2010;51:3067–3075) DOI:10.1167/iovs.09-4313

Behçet’s disease (BD) is a multisystem inflammatory disease characterized by recurrence of oral ulcers, uveitis, genital ulcers, and skin lesions.1–3 Generally, ocular involvement is observed in 70% of patients with BD and is the initial manifestation in approximately 10% of BD patients.4 The typical form of ocular involvement is relapsing and self-limiting uveitis, which sometimes leads to blindness in patients with repeat severe ocular inflammation. Because biomarkers for BD have not been identified, the disease is still diagnosed according to clinical symptoms.5,6 At the onset of the disease, it is sometimes difficult to distinguish BD from other forms of uveitis that manifest acute and nongranulomatous ocular inflammation, especially when ocular inflammation precedes other systemic symptoms of BD.

Although various primary immune abnormalities involving genetic elements, infectious agents, neutrophil dysfunction, and autoimmune components have been implicated in BD,7–12 the pathogenesis of this disease remains uncertain. In studies exploring autoimmune responses to autoantigens in BD, S antigen (S-Ag),13,14 interphotoreceptor retinoid binding protein (IRBP),15 heat-shock protein 60,16 α-enolase,16 α-tropomyosin,17,18 esterase D,19 and selenium binding protein20 are the major autoantigens identified. Lymphocyte proliferation assays with peripheral blood lymphocytes are commonly used to detect cellular immune response to antigens. Although these assays are capable of providing evidence of immune responses, they do not provide detailed and substantial information on the status of progression of immune responses.

Both innate and adaptive immune responses have been suggested to play a pathogenic role in BD.21–25 Aberrant cellular immunity, such as T-cell-mediated autoimmunity or Th1/Th2 imbalance, may be crucial in the pathogenesis of BD.26–29 In addition, recent studies have shown that immune responses mediated by Th17 cells, which are characterized by the production of high levels of IL-17, IL-6, and TNF-α but a low level of INF-γ,30 are upregulated in BD.31,32 Zhao et al.26 have indicated that peripheral blood mononuclear cells from BD patients with active uveitis respond to S-Ag challenge to produce INF-γ and TNF-α but not IL-2, IL-4, or IL-17. Therefore, in the present study, we measured cytokines related to innate and acquired immune responses mediated by Th1, Th2, and Th17 cells specific for IRBP and S-Ag in BD patients and attempted to characterize the major immune responses involved in the pathogenesis of uveitis in BD.

SUBJECTS, MATERIALS, AND METHODS

Subjects

Thirty-three patients who visited the Uveitis Clinic of Tokyo Medical University Hospital between January and December 2008 and received diagnoses of BD according to the criteria of the International Study Group for BD were enrolled in this study. Twenty-three healthy subjects were enrolled as controls. The profiles of the patients and healthy controls are shown in Table 1. BD patients were classified into “active uveitis” or “uveitis in remission” according to the presence or absence of active ocular inflammation on the day of sample collection. Active uveitis was defined as the presence of at least one of the
following ocular inflammatory signs observed by ophthalmoscope: (1) 2+ infiltrating cells in the anterior chamber; (2) worse vitreous opacity; (3) retinal exudates. Uveitis in remission was defined as the absence of any of the above signs. The protocol for this study adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review board of Tokyo Medical University. Written informed consent was obtained from all participating patients and controls.

**Preparation of IRBP and S-Ag**

Fresh swine retinas were homogenized in 0.05 M disodium monopotassium phosphate buffer (PB; pH 7.6). After centrifugation (48,000g for 10 minutes), the supernatant was collected. Saturated ammonium sulfate (pH 7.2) was added to the supernatant until 50% saturation was achieved, and the mixture was left overnight at 4°C. After centrifugation, the precipitate was dissolved in PB and was used as the crude antigen preparation. IRBP was isolated from the crude antigen preparation according to the method of Dorey et al. Briefly, to obtain better quality of IRBP, the crude preparation was purified successively by concanavalin A-Sepharose affinity chromatography and ion exchange high-performance liquid chromatography. S-Ag was also isolated from the same crude preparation essentially according to the method of Dorey et al. Briefly, the crude preparation was successively subjected to gel filtration (Sephadex G-200; Pharmacia, Uppsala, Sweden) and adsorption chromatography on hydroxyapatite-agarose.

**Isolation of Peripheral Blood Mononuclear Cells**

Twenty milliliters of peripheral venous blood was drawn into a heparinized tube from all BD patients and controls. Peripheral blood mononuclear cells (PBMCs) were isolated immediately by density gradient centrifugation (Ficoll-Hypaque; Pharmacia Biotech, Shanghai, China) and suspended at 2 × 10⁶ cells/mL in RPMI 1640 medium supplemented with 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin (all from BioWhittaker, Walkersville, MD), 1 × 10⁻⁴ M 2-ME (Sigma Chemical Co., St. Louis, MO), and 10% fetal calf serum (Sigma Chemical Co.).

**Cytokine Production Assay**

Fresh PBMCs (2 × 10⁵) were added to micro wells in triplicate and incubated with IRBP, S-Ag, or tuberculin purified protein derivative (PPD) at a concentration of 0, 1, 5, or 10 μg/mL for 48 hours. Supernatants were collected, and the concentrations of IL-2, IL-4, IL-6, IL-10, IFN-γ, IL-12, IL-17, and TNF-α were measured with a cytokine kit (Human Th1/Th2 Cytokine Cart Kit II; BD Pharmingen, San Diego, CA), and the IL-17 concentration was measured with an ELISA kit (ELISA Ready-SET-Go kit; eBioscience, San Diego, CA) according to the protocols recommended by the manufacturers. The lower detection limit was 3 pg/mL for the IL-2, IL-4, IL-6, IL-10, and TNF-α assays, 8 pg/mL for the IFN-γ assay, and 15.6 pg/mL for the IL-17 assay.

**Statistical Analysis**

Data were analyzed with statistical software (JMP 5; SAS Institute Inc., Cary, NC). Data were expressed as mean ± SD. Statistical analysis was performed using paired or unpaired t-test and ANOVA test. *P < 0.05 was considered significant; significance is denoted by asterisks in the figures.

**RESULTS**

**Th1, Th2, and Th17 Cytokine Production by PBMCs from Behc¸et’s Patients and Healthy Controls Stimulated with IRBP or S-Ag**

PBMC samples obtained from BD patients were stimulated with various concentrations of IRBP or S-Ag, and Th1 (IL-2, IFN-γ, and TNF-α), Th2 (IL-4, IL-6, and IL-10), and Th17 (IL-17) cytokine production was measured (Fig. 1). IL-2 is a growth factor of Th0 and Th1 cells, and IL-2 production correlates best with T-cell proliferation response, whereas IL-4 promotes the differentiation and proliferation of Th2 cells. In the present study, significant production of IL-2 or IL-4 was not observed with IRBP or S-Ag stimulation of PBMCs in BD patients. However, both IRBP and S-Ag stimulated significant production of IFN-γ, IL-6, and IL-17 in a dose-dependent manner, and significant production of TNF-α was observed by stimulation with IRBP. Surprisingly, both IRBP and S-Ag stimulation also induced dose-dependent production of IL-10, which is a Th2 cytokine known to suppress the development of uveitis in animal models. Similar results of cytokine profiles were observed with PBMCs obtained from healthy controls who never had uveitis (Fig. 2). Although no significant production of IL-2 and IL-4 was observed, both IRBP and S-Ag induced significant production of IL-6, IL-10, IL-17, and IFN-γ, but only IL-2 was able to stimulate TNF-α production.

**Comparison of Cytokine Production between Behc¸et’s Patients and Healthy Controls Stimulated with IRBP or S-Ag**

Subsequently, IL-6, IL-10, IL-17, IFN-γ, and TNF-α production by PBMCs of BD patients were compared with that of healthy controls. When stimulated with IRBP, the levels of IL-6, IL-17, and IFN-γ produced by PBMCs of BD patients were significantly higher than those of healthy controls (Fig. 3). However, production of TNF-α, which is a proinflammatory cytokine known to be involved in the development of uveitis, was comparable between BD patients and controls. Furthermore, production of IL-10 was not significantly different between BD and controls.

On stimulation with S-Ag, apart from a higher IL-6 production by PBMCs of BD patients compared with that of healthy controls, no significant differences in other cytokine levels were observed between BD patients and controls (Fig. 4).

| Table 1. Characteristics of Patients with Behc¸et’s Disease and Healthy Controls |
|-----------------|-----------------|-----------------|
| **Behc¸et’s Patients** | **Healthy Controls** |
| Number | 33 | 23 |
| Male/female | 21/12 | 13/10 |
| Age, y | 42.2 ± 12.8 | 47.3 ± 19.1 |
| Uveitis duration, mo | 3.8 ± 4.2 | — |
| Uveitis activity, active/remission | 17/16 | — |
| HLA-B51 positive | 12 | — |
| HLA-A26 positive | 12 | — |
| **Ocular findings** |
| Iridocyclitis | 33/33 (100%) | — |
| Hyopyon | 12/33 (36.4) | — |
| Dense vitreous opacity | 30/33 (90.9) | — |
| Retinal exudates† | 14/33 (42.4) | — |
| Retinal vasculitis‡ | 29/33 (87.9) | — |
| **Other BD symptoms** |
| Oral aphtha | 33/33 (100) | — |
| Dermatitis | 23/33 (69.7) | — |
| Genital ulcer | 7/33 (21.2) | — |
| Arthritis | 7/33 (21.2) | — |
| **Systemic treatment** |
| Colchicine | 29/33 (87.9) | — |
| Steroid | 4/33 (12.1) | — |
| Cyclosporine | 1/33 (3.0) | — |
| Infliximab | 7/33 (21.2) | — |

* Positive rates in parentheses.
† Retinal exudates with hemorrhage within retinal vascular arcade.
‡ Diffuse retinal vasculitis observed clinically and disclosed by fluorescein angiography.
FIGURE 1. Production of Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-6, IL-10), and Th17 (IL-17) cytokines by PBMCs of BD patients when stimulated with IRBP and S-Ag. PBMCs were isolated from 33 BD patients. Each fresh PBMC sample was incubated with IRBP or S-Ag at a concentration of 0, 1, 5, or 10 μg/mL for 48 hours. Supernatants were collected, and the amounts of IL-2 (A), IL-4 (B), IL-6 (C), IL-10 (D), IL-17 (E), IFN-γ (F), and TNF-α (G) were measured. Data are expressed as mean ± SD for all BD patients. *Significant difference compared with the cytokine level measured in supernatants of PBMCs without antigen stimulation (P < 0.05).
FIGURE 2. Production of Th1, Th2, and Th17 cytokines by PBMCs of healthy controls when stimulated with IRBP and S-Ag. PBMCs were isolated from 23 healthy controls. Each fresh PBMC sample was incubated with IRBP or S-Ag at a concentration of 0, 1, 5, or 10 μg/mL for 48 hours. Supernatants were collected, and the amounts of IL-2 (A), IL-4 (B), IL-6 (C), IL-10 (D), IL-17 (E), IFN-γ (F), and TNF-α (G) were measured. Data are expressed as mean ± SD for all healthy controls. *Significant difference compared to each cytokine level measured in supernatants of PBMCs without antigen stimulation (P < 0.05).
From these results, it is conceivable that IRBP-induced IL-6, IL-17, and IFN-γ* production, as well as S-Ag–stimulated IL-6 production, may be related to the development of uveitis in BD patients.

Comparison of Cytokine Production in Behçet’s Patients and Healthy Controls Stimulated with IRBP, S-Ag, or PPD

Given that all subjects enrolled in this study had received BCG vaccination against tuberculosis, they should have had immune responses to PPD. Therefore, the production of IL-6, IL-10, IL-17, IFN-γ, and TNF-α by PBMCs when stimulated with IRBP, S-Ag, and PPD was compared in BD patients and healthy controls. Surprisingly, this cytokine production by PBMCs on stimulation with IRBP and S-Ag was comparable with that on PPD stimulation in BD patients or healthy controls (Fig. 5). In BD patients, IL-6 production by PBMCs was significantly higher when stimulated with PPD or S-Ag than with IRBP, but there was no significant difference in IL-10 production between IRBP, S-Ag, and PPD stimulation. Conversely, IL-17 production was dominantly induced by IRBP stimulation and was significantly higher than S-Ag or PPD stimulation. IFN-γ production was also significantly higher on stimulation with IRBP than with S-Ag. On the other hand, TNF-α production was not significantly different between IRBP and S-Ag stimulation but was significantly higher on stimulation with PPD than with S-Ag. Similar results were obtained using PBMCs obtained from healthy controls. IL-6 production was significantly higher when stimulated with PPD or S-Ag than with IRBP; conversely, IL-17 and IFN-γ production was significantly higher when stimulated with IRBP than with S-Ag. TNF-α production was significantly higher on stimulation with PPD than with S-Ag. When PBMCs of BD patients and healthy controls were stimulated with PPD, the amounts of cytokine produced were apparently higher in BD patients than in healthy controls, but the differences were not statistically significant.

Comparison of Cytokine Production in Behçet’s Patients with Active Uveitis and Behçet’s Patients with Uveitis in Remission

To attempt to identify the cytokines involved in ocular inflammation induced by uveitis, cytokines produced by PBMCs were compared between BD patients with active uveitis and patients with uveitis in remission. When stimulated with IRBP, only IFN-γ was produced at significantly higher levels in BD patients with active uveitis than in BD patients with uveitis in remission, whereas other cytokines involved in the development of uveitis, including IL-10, were not significantly different between the two groups of BD patients (Fig. 6).

On stimulation with S-Ag, all the cytokines examined showed comparable levels between BD patients with active uveitis and those with uveitis in remission, at all concentrations tested (Fig. 7).
Effects of HLA-B51 or HLA-B26 on Cytokine Production by PBMCs of Behçet’s Patients

HLA-B51 and HLA-B26 are known to be susceptible genes to BD.35–37 Therefore, IL-6, IL-10, IL-17, IFN-γ, and TNF-α production by PBMCs on autoantigen stimulation were compared between BD patients with (n = 12) and without (n = 21) HLA-B51 and between BD patients with (n = 12) and without (n = 21) HLA-A26. For the HLA-B51 allele (Fig. 8), IRBP-stimulated TNF-α production was significantly higher in HLA-B51-positive than in HLA-B51-negative patients, whereas no significant differences between the two groups were observed in other cytokine levels stimulated with either IRBP or S-Ag. Although IRBP-stimulated IFN-γ production was apparently lower in HLA-A26-positive than in HLA-A26-negative patients, there was no significant difference (Fig. 9). Other cytokine levels stimulated with either IRBP or S-Ag were also not significantly different between HLA-A26-positive and HLA-A26-negative patients.

**DISCUSSION**

 Immune responses to retinal autoantigens, including IRBP and S-Ag, have been demonstrated in BD patients with uveitis.15–36 In the present study, PBMCs from healthy controls who never had uveitis also produced cytokines on stimulation with IRBP and S-Ag, suggesting that autoreactivity to retinal autoantigens is normal in humans. In animal experiments, lymphocytes obtained from nonimmunized normal rodents are not capable of producing any cytokine in vitro on stimulation with IRBP or S-Ag. As in the induction of uveitis, cytokine production by lymphocytes requires immunization with IRBP or S-Ag to activate and expand the IRBP-specific or S-Ag-specific T cells. Given that all BD patients and controls studied had a history of BCG vaccination, it is reasonable that PBMCs stimulated by PPD produced IL-6, IL-10, IL-17, IFN-γ, and TNF-α. However, IRBP and S-Ag stimulated the production of IL-6, IL-10, IL-17, IFN-γ, and TNF-α by PBMCs of BD patients and healthy controls as potently as the immunization antigen PPD. In addition, when we studied sarcoidosis patients with uveitis, who showed conversion from tuberculin-positive to tuberculin-negative, IFN-γ production by their PBMCs on stimulation with IRBP or S-Ag was inhibited (unpublished data, 2009). Therefore, it is conceivable that in humans, IRBP- and S-Ag-specific T cells are spontaneously sensitized and that everyone is potentially susceptible to uveitis when challenged by specific genetic or environmental factors. As a preliminary attempt to examine this possibility, we analyzed the associations between the ages of BD patients or healthy subjects or their durations of uveitis and the levels of individual cytokines produced by PBMC on stimulation with IRBP or S-Ag. However, no significant correlation was detected (data not shown). This hypothesis could be examined in detail in further studies by analyzing the response of lymphocytes collected from cord blood.

In BD patients and healthy controls, IL-17, IFN-γ, and TNF-α production was significantly higher when stimulated with IRBP

**FIGURE 5.** Comparison of cytokine productions by PBMCs of BD patients and healthy controls between IRBP, S-Ag, and PPD stimulation. IL-6 (A), IL-10 (B), IL-17 (C), IFN-γ (D), and TNF-α (E) produced by PBMCs of BD patients and healthy controls were compared between stimulation with 10 μg/mL of IRBP (white bar), S-Ag (black bar), and PPD (gray bar). Data are expressed as mean ± SD for all BD patients (n = 10) and healthy controls (n = 7). *Significant difference in cytokine level between IRBP, S-Ag, and PPD stimulation (P < 0.05).

**FIGURE 6.** Comparison of IRBP-stimulated cytokine production between BD patients with active uveitis and those with uveitis in remission. IL-6 (A), IL-10 (B), IL-17 (C), IFN-γ (D), and TNF-α (E) produced by PBMCs on stimulation with IRBP were compared between BD patients with active uveitis (n = 17; ○) and those with uveitis in remission (n = 16; ●). Data are expressed as mean ± SD for BD patients in each group. *Significant difference in cytokine level between active uveitis and uveitis in remission (P < 0.05).
than with S-Ag, whereas IL-6 production was significantly higher when stimulated with S-Ag than with IRBP. IL-6 is produced by many types of cells, including Th2 cells, Th17 cells, monocytes, and fibroblast. In addition, it is unclear whether IRBP-specific and S-Ag-specific T cells differentiate into Th1, Th2, Th17, or other cell types in BD patients and in healthy controls. Therefore, our findings suggest at least that immune responses mediating IL-6 production prefer S-Ag to IRBP stimulation. Alternatively, it is likely that IRBP-specific T cells differentiate toward Th1 and Th17 cells in BD patients and controls.

In this study, IRBP-stimulated IFN-γ and IL-17 production by PBMCs was significantly higher in BD patients than in healthy controls, indicating Th1 and Th17 polarization are compatible with previous reports that Th1 and Th17 cytokines contribute to the pathogenesis of BD. Numerous studies on endogenous uveitis have been performed using experimental autoimmune uveoretinitis (EAU) induced by immunization with IRBP or S-Ag, or adoptive transfer of IRBP- or S-Ag-specific T cells, and have elucidated extensively the pathogenic mechanisms. A recent study on the role of retinal antigen-specific Th1 and Th17 cells in the pathogenesis of EAU showed that the role of the Th17 effector is redundant with Th1, and each effector phenotype by itself is sufficient to induce EAU. However, IL-10, which is a Th2 cytokine and inhibits the development of uveitis, is produced to the same extent by PBMCs of BD patients and healthy controls. This result suggests that Th2 type responses to IRBP or S-Ag is unlikely to be involved in the development of uveitis in BD.

Although IL-6, IL-17, and IFN-γ production was higher in BD patients than in healthy controls, only IRBP-stimulated IFN-γ production was significantly increased in BD patients with active uveitis compared with those with uveitis in remission. IFN-γ is considered to be a major effector cytokine in the pathogenesis of autoimmunity. IFN-γ is predominantly related to delayed-type hypersensitivity responses by the upregulation of HLA-DR activation in a variety of cells, including dendritic cells and macrophages that generate IL-12 and further facilitate Th1 cell development. Elevated levels of IFN-γ have been found in the aqueous humor and serum of BD patients compared with healthy controls, and the frequencies of Th1 cytokine-producing cells are also increased in patients with active BD. Considering these past reports and the present findings, it is possible that IFN-γ production by PBMCs stimulated with IRBP may be an index of uveitis activity in BD patients.

It has been reported that the TNFB2 allele is associated with HLA-B51 among BD patients and that the frequency of this allele is significantly higher than in BD patients without uveitis and healthy controls. TNF-α production is high in BD patients, and the TNFB2 allele has been found to be associated with high TNF-α production by leukocytes. Therefore, the present result that HLA-B51 was associated with IRBP-stimulated TNF-α production by BD patients might be related to TNFB2.
inflammation in the eye. Immune responses are potentially involved in the progression of or reflect ongoing uveitis, both IRBP- and S-Ag–mediated immunity in Behc¸et disease. Isotype between HLA-A26–positive and HLA-A26–negative BD patients. Data are expressed as mean ± SD for BD patients in each group. *Significant difference in cytokine level between HLA-A26 positive and negativity (P < 0.05).

In summary, the present study demonstrated that cytokines related to Th1-, Th2-, and Th17-mediated immune responses were produced by PBMCs stimulated with IRBP or S-Ag in BD patients and healthy controls. However, IL6 produced by S-Ag–specific T cells, as well as IL-6, IFN-γ, and IL-17 produced by IRBP-specific T cells, were remarkably augmented in BD patients compared with healthy controls. In particular, IFN-γ produced by IRBP-specific T cells was significantly increased in BD patients during the active phase of uveitis. Although it is difficult to determine whether these cytokines produced by PBMCs of BD patients contribute to the development of uveitis or reflect ongoing uveitis, both IRBP- and S-Ag–mediated immune responses are potentially involved in the progression of inflammation in the eye.

References


