Higher Expression of Toll-like Receptors 2, 3, 4, and 8 in Ocular Behcet's Disease

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PURPOSE. To investigate the role of Toll-like receptors (TLRs) 2, 3, 4, and 8 in the pathogenesis of Behcet's disease (BD).

METHODS. Sixteen patients with active ocular BD and 16 healthy volunteers were included in this study. Total RNA was isolated from PBMCs to determine mRNA levels of TLRs, including TLR2, TLR3, TLR4, and TLR8. Cell surface receptor activity of these TLRs was investigated by FACS analysis. Monocytes and naïve T cells from patients and controls were cultured with or without TLR ligands, such as LPS, PGN, R848, or PolyI:C. Culture supernatants were collected and IL-17, IL-1β, and IL-23 were analyzed by ELISA.

RESULTS. A markedly higher expression at the mRNA and protein level of TLR2, TLR3, TLR4, and TLR8 was observed in active BD patients as compared with controls. Significantly higher levels of IL-1β and IL-23 were detected in the supernatants of monocytes stimulated with LPS or PGN. A significantly higher level of IL-17 was observed in the supernatants of naïve T cells and monocytes stimulated with LPS or PGN in BD patients as compared with controls. Upon stimulation with R848 or PolyI:C, the levels of IL-17 in the supernatants of naïve T cells and monocytes and IL-23 levels in the supernatants of monocytes were not different between BD patients and controls.

CONCLUSIONS. A higher expression of TLRs may be involved in the pathogenesis of BD.

Keywords: Behcet's disease, Toll-like receptor, IL-17, TLR ligands

Behcet's disease (BD) is characterized by recurrent uveitis, oral aphthae, genital ulcers, and skin lesions.[1,2] The etiopathogenesis of BD is still not completely understood. An autoinflammatory response is considered to be involved in the pathogenesis of this disease, as evidenced by increased levels of cytokines and transcription factors of Th1 and Th17 cells in patients with active BD.[3–7] However, *Staphylococcus aureus* and *Streptococcus oralis* have been identified in the skin lesions of BD patients.[8,9] Ayaslioglu et al.[10] found a higher IgA seropositivity to *Chlamydia pneumoniae* in BD patients. Recent studies have suggested that improved oral hygiene may be associated with the observed decline in the incidence of BD in Japan.[11] The results mentioned above suggest that microorganisms may be involved in the development of BD.

It has been well demonstrated that antigen-presenting cells (APCs), including dendritic cells, can present microorganisms or pathogens to T cells and induce the differentiation and proliferation of effector T cells.[12] Toll-like receptors (TLRs) play a critical role in innate immunity against pathogens, and currently 10 TLRs have been identified in humans. They have been named TLR1 to 10, of which TLR2, TLR3, TLR4, and TLR8 have been well studied.[13–17] TLR2 recognizes a wide spectrum of microbial molecules, including gram-positive and gram-negative bacteria, parasites, fungi, and viruses. TLR3 is a receptor that recognizes double-stranded RNA (dsRNA) molecules such as polycytidylic acid [Poly(I:C)], thereby mimicking viral dsRNA, which is a

molecular pattern found in most viruses. TLR4 interacts with a component of the outer membrane of gram-negative bacteria and TLR8 plays an important role in the recognition of viral single-stranded RNA.[18] TLRs on APCs can act as a bridge between infection and an autoimmune response that follows. Interaction of APC with TLR ligands results in the secretion of a number of proinflammatory cytokines, such as IL-1beta, IL-6, IL-23, and TNF-alpha, which can induce the differentiation of naïve CD4^{+} T cells into Th17 cells.[19,20] Various studies have shown increased levels of IL-17 and IL-23 in patients with active BD, supporting an autoinflammatory pathogenesis of this disease.[5,21]

As yet, the role of TLRs in the development of BD is not completely clear and was therefore the subject of the study presented here. We investigated the levels of several TLRs (TLR2, TLR3, TLR4, and TLR8) and studied the in vitro response of BD patients to TLR ligands, including lipopolysaccharide (LPS), peptidoglycan (PGN), R848, or PolyI:C. Our results show an upregulated TLR response in BD, which might provide further support for the role of infection in the development of this disease.

MATERIALS AND METHODS

Subjects

Sixteen patients (nine men and seven women, with an average age of 34.5 years) with active BD were included in this study.

Sixteen healthy individuals (nine men and seven women, with an average age of 33 years) acted as controls. Two sets of experiments were done, each comprising eight patients and eight controls. Patients and controls were included between June 2008 and May 2011. The diagnosis of BD was based on the criteria of the International Study Group for BD.[22] At the time of their visit to our clinic, all patients had active ocular inflammation, as evidenced by decreased vision (100%), hypopyon (25%), cells and flare in the anterior chamber (100%), keratic precipitates (100%), vitreous cells (50%), or retinal vasculitis (100%). The extraocular findings included recurrent oral aphthous lesions (100%), recurrent genital ulcers (43.7%), multiform skin lesions (62.5%), and arthritis (31.2%). No immunosuppressive agents or prednisone were given to these active BD patients at least 2 weeks before visiting us and blood sampling. Written and informed consent was received from all patients and healthy controls. The study met the tenets of the Declaration of Helsinki and was approved by our Clinical Ethical Research Committee.

Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples from BD patients and controls by Ficoll-Hypaque density-gradient centrifugation. Naïve T cells were isolated from PBMCs by naive $CD4^+$ T-cell isolation kit (purity > 90%; Miltenyi Biotec, Palo Alto, CA), according to the manufacturer's instructions. CD14-positive monocytes were isolated by human CD14 microbeads (purity > 90%; Miltenyi Biotec), according to the manufacturer's instructions. To investigate the effect of the four TLR ligands on the production of IL-1β and IL-23 by monocytes, we cultured monocytes from BD patients and healthy controls with recombinant human granulocyte macrophage colony-stimulating factor (100 ng/mL; R&D Systems, Minneapolis, MN) and and recombinant interleukin 4 (rIL-4; ng/mL, R&D Systems); half of the culture medium, including recombinant cytokines, was refreshed on day 3. LPS has been well investigated in the proinflammatory cytokine production by monocytes.[23,24] In this study, we used LPS as a control to study the role of PGN, R848, or PolyI:C in the production IL-1β and IL-23 by monocytes. Cell cultures were then incubated with one of the stimulators, including LPS, PGN, R848, or PolyI:C, for another 48 hours on day 5. Supernatants were collected for IL-1β and IL-6 measurement by ELISA. To detect whether TLR ligands, including LPS, PGN, R848, and PolyI:C, could induce IL-17 production in BD patients and healthy controls, we cocultured the same number of monocytes and naïve T cells stimulated with anti-CD3 antibody (5 μg/mL; eBioscience, San Diego, CA) together with one of the following stimulators: LPS from *Escherichia coli* 0111:B4 (100 ng/mL, Sigma-Aldrich, St. Louis, MO), PGN from *S. aureus* (5 μg/mL; Fluka, Buchs, Switzerland), R848 (2.5 μg/mL; Alexis Biochemicals, San Diego, CA), or PolyI:C (100 ng/mL, Sigma-Aldrich) for 3 days. Supernatants were subsequently obtained for IL-17 measurement by ELISA.

RT-PCR

Total RNA was extracted from PBMCs of controls and patients by the RNAprep Cell Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Reverse transcription of RNA was performed using the Superscript III Reverse Transcriptase system (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed on the AB 7500 Fast System (Applied Biosystems, Foster City, CA) using the Quanti Tect SYBR Green PCR kit (Applied Biosystems). The primer sequences used were: β-actin forward, 5'-GGATGCAGAAGGA-GATCAC TG-3', reverse, 5'-CGATCCACACGGAGTACTTG-3'. The primer sequences of TLR2, TLR3, TLR4, and TLR8 were used as described elsewhere.[15] The expression of each gene was normalized to the expression of β-actin using the $2^{-\Delta\Delta CT}$ method.[25]

Flow Cytometric Analysis

The flow cytometric cell surface staining method was used for TLR2 and TLR4. Briefly, PBMCs were washed and then stained with the antihuman CD4-PE-Cy7, antihuman CD14-APC, and corresponding TLR antibodies or control antibodies. The intracellular staining method was used for TLR3 and TLR8, whereby PBMCs were first stained with antihuman CD4-PE-Cy7 and antihuman CD14-APC for 30 minutes, fixed, and permeabilized with BD-Cytofix/Cytoperm buffer (BD PharMingen, San Diego, CA).

Then the cells were incubated with corresponding TLR antibodies or corresponding isotypic control antibodies. Fluorescent antibodies (antihuman CD4-PE-Cy7, antihuman CD14-APC, antihuman TLR4-PE, antihuman TLR3-PE, antihuman TLR2-FITC, and antihuman TLR8-FITC) were obtained from BD Biosciences (BD Biosciences, Sunnyvale, CA). Samples were analyzed on a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences). The results were expressed as the percentage difference compared with isotypic control (IC) using the following formula: (mean fluorescence intensity [MFI] of TLR – MFI of IC)/MFI of IC × 100%.

ELISA

The concentration of IL-17 in the supernatants of cocultured naïve T cells and monocytes was measured using the human Duoset ELISA development kit (R&D Systems), which has a detection limit of 15 pg/mL. The levels of IL-1β in the supernatants of monocytes were detected by a human Duoset ELISA development kit (R&D Systems) with a detection limit of 3.9 pg/mL. The concentration of IL-23 was detected using a human IL-23 (p19/p40) ELISA Ready-SET-Go Kit (eBioscience) with a detection limit of 31 pg/mL.

Statistical Analysis

The ANOVA, independent-samples *t*-test, Mann-Whitney *U* test, and the paired-samples *t*-test were applied using SPSS 10.0 software (IBM SPSS Inc., IBM Corporation, Chicago, IL). Data are shown as mean ± SD. *P* values less than 0.05 were considered significant.

RESULTS

Increased Expression of TLRs at the mRNA and Protein Level in Active BD Patients

We isolated total RNA from PBMCs of BD patients and controls and evaluated the mRNA levels of TLR2, TLR3, TLR4, and TLR8 using RT-PCR. The results showed that mRNA levels of the four tested TLRs in PBMCs of active BD patients were significantly higher than that observed in controls (Fig. 1). We further tested the receptor positivity of these TLRs in PBMCs, and isolated $CD4^+$ T cells and monocytes in active BD patients and controls using FACS analysis. The results showed that the cell surface receptor activity of these TLRs in BD was also markedly higher than that seen in controls in all three types of cells investigated (Fig. 2).

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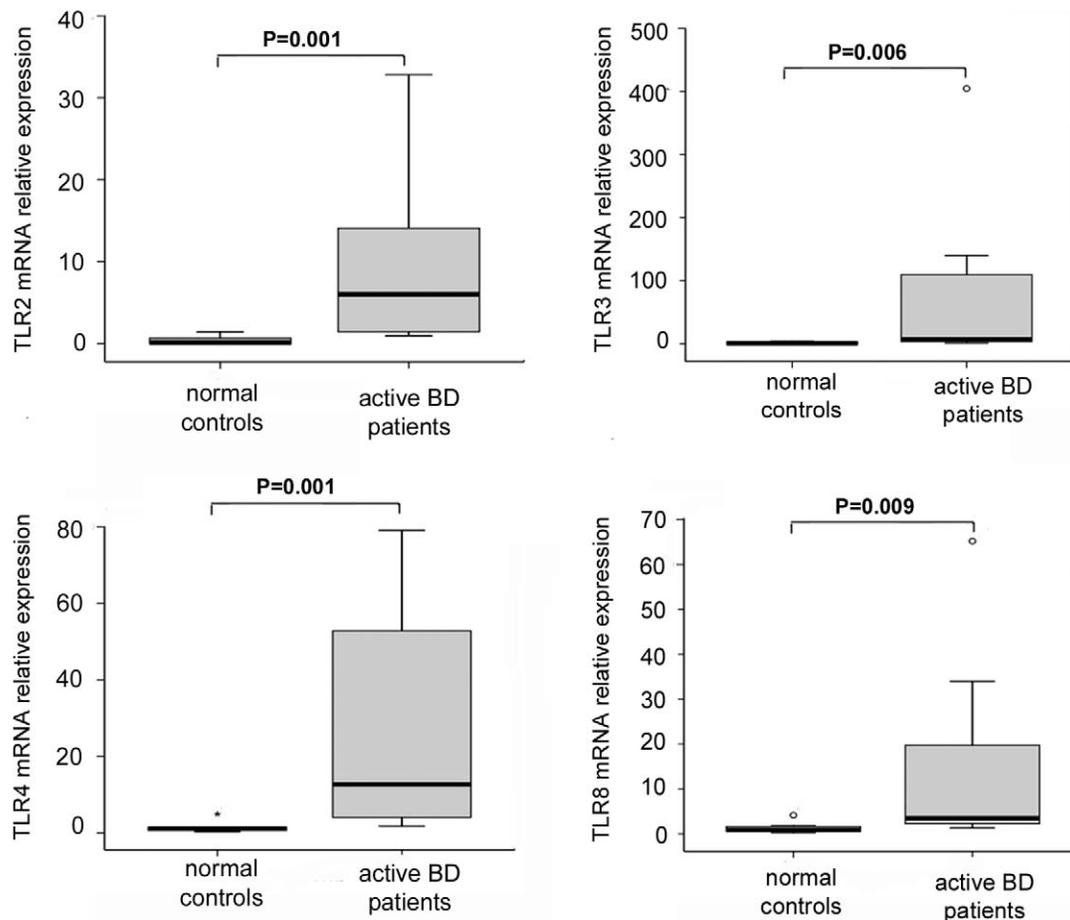
FIGURE 1. mRNA levels of TLR2, TLR3, TLR4, and TLR8 in PBMCs from active BD patients ($n = 8$) and healthy controls ($n = 8$) were measured by real-time PCR. Results are normalized to β-actin. Data are presented with box-and-whisker plots. Independent-samples t-test or Mann-Whitney U test were applied for statistical analysis.

Ligands of TLR Stimulate the Production of IL-1β and IL-23 by Monocytes in Active BD Patients

Because IL-1β and IL-23 are two important cytokines that are critical to the induction of IL-17 production by Th17 cells,[26] we performed a set of experiments to investigate whether the four TLR ligands could induce the production of these two cytokines and whether there was a difference between BD patients and healthy controls. All TLR ligands tested markedly increased the production of IL-1β by monocytes both from BD patients and from controls. However, significantly higher levels of IL-1β were produced by monocytes from BD patients as compared with controls. No significant difference was observed with regard to the stimulation effect on IL-1β production among LPS, PGN, R848, and PolyI:C in controls. No significant difference concerning the IL-1β production by monocytes without TLR ligand stimulation was observed between BD patients and controls. In the BD patients, a stronger effect was observed when cells were stimulated with PGN and LPS as compared with R848 and PolyI:C (Fig. 3).

The levels of IL-23 were under the detection limit in the supernatants of monocytes from controls on stimulation with any one of the four TLR ligands and in the supernatants of monocytes from BD patients stimulated with R848 or PolyI:C. However, IL-23 became detectable in the supernatants of monocytes from BD patients stimulated with PGN or LPS. A stronger stimulatory effect on IL-23 production was observed

on stimulation with PGN as compared with LPS ($P = 0.001$) (Fig. 4).

Ligands of TLR Significantly Promote the Production of IL-17 by Naïve T Cells Cocultured With Monocytes in Active BD Patients

To study whether there was a difference in the response to pathogens between BD patients and controls, naïve T cells and monocytes were cultured alone or respectively with LPS, PGN, R848, or Poly I:C, and the concentration of IL-17 in supernatants of these cells was determined. The results showed that the naïve T cells and monocytes cultured alone produced a basal level of IL-17 in both BD patients and controls. Stimulation with any one of the four ligands significantly stimulated IL-17 production by naïve T cells in BD patients and controls. A stronger effect was observed on stimulation with LPS and PGN in BD patients as compared with controls. LPS showed a significantly higher stimulation on the production of IL-17 as compared with R848 and Poly I:C in BD patients (Fig. 5).

DISCUSSION

Our study showed a markedly higher expression of TLR2, TLR3, TLR4, and TLR8 by PBMCs, CD4^{+} T cells, or monocytes obtained from BD patients as compared with controls.
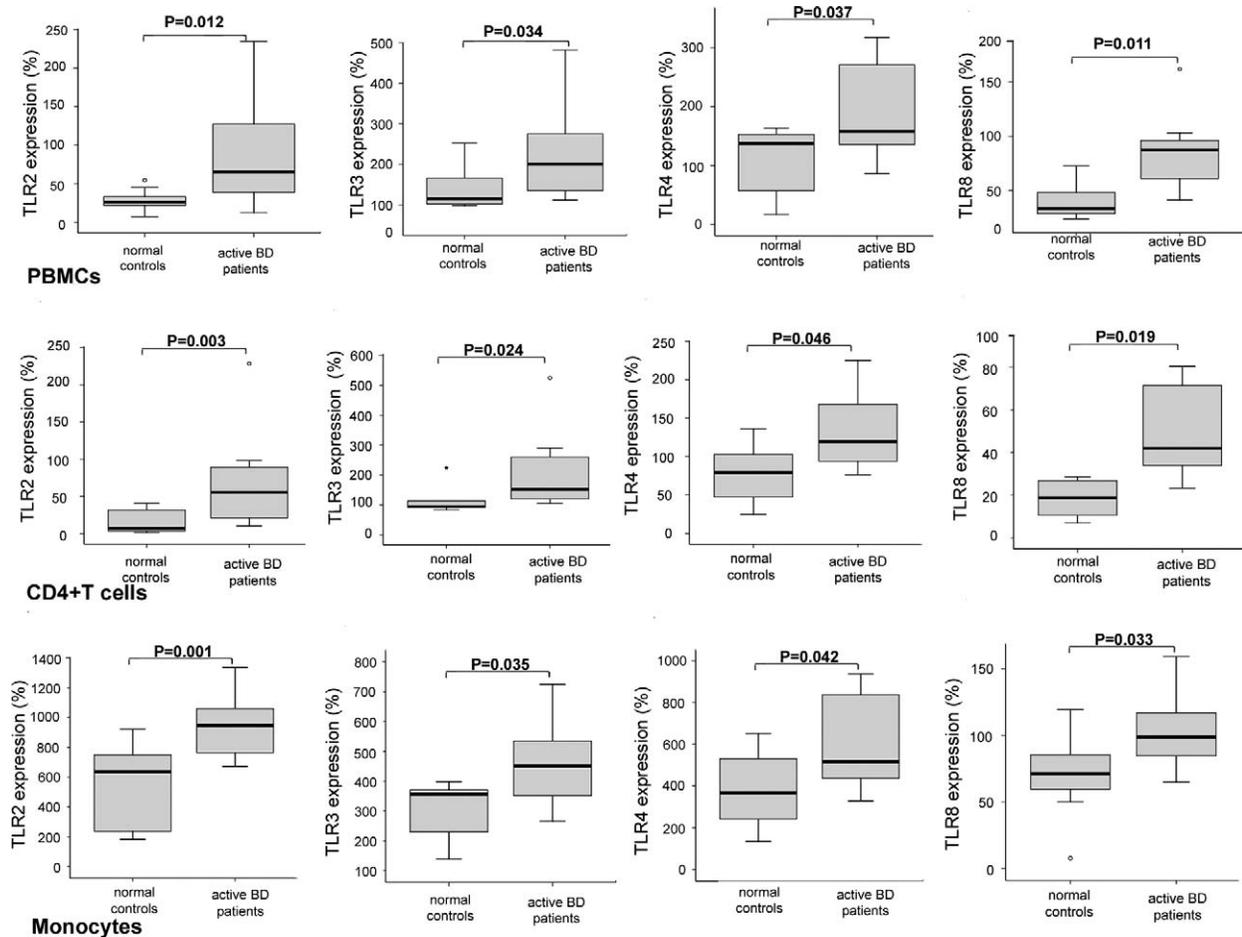
FIGURE 2. Cell surface receptor activity of TLR2, TLR3, TLR4, and TLR8 was detected by FACS analysis in PBMCs, CD4⁺ T cells, and monocytes from active BD patients ($n = 8$) and controls ($n = 8$). Results are expressed as percentage difference compared with isotypic controls and presented as box-and-whisker plots. The Mann-Whitney U test or independent-samples t-test was used to assess the differences in expression among active BD patients and healthy controls.

Significantly higher levels of IL-1β and IL-23 were produced by monocytes from BD patients stimulated with known TLR ligands, such as LPS and PGN. Correspondingly, markedly higher levels of IL-17 were produced by cocultured naive T cells and monocytes stimulated with any one of the four TLR ligands, including LPS, PGN, R848, and poly I:C in BD patients and in controls. Furthermore, higher levels of IL-17 were
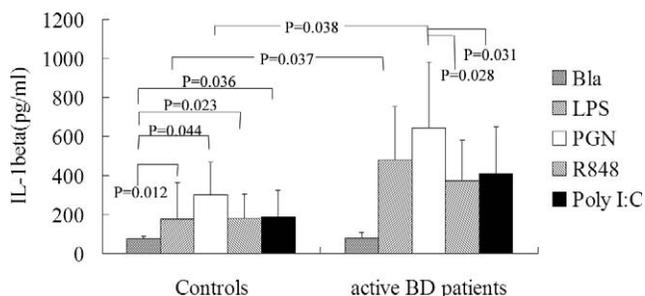
FIGURE 3. IL-1β production by monocytes stimulated with LPS, PGN, R848, or PolyI:C from active BD patients ($n = 8$) and controls ($n = 8$). The *dark triangle* indicates that the levels of IL-1β in the supernatants of monocytes stimulated with LPS, PGN, R848, or PolyI:C were significantly higher as compared with the unstimulated control group ($P < 0.001$). ANOVA was used for statistical analyses. Data are expressed as mean ± SD.

observed in the supernatant of cocultured naive T cells and monocytes from BD patients stimulated with LPS and PGN as compared with the other ligands. Taken together, these results suggest that a higher expression of TLR is associated with BD.
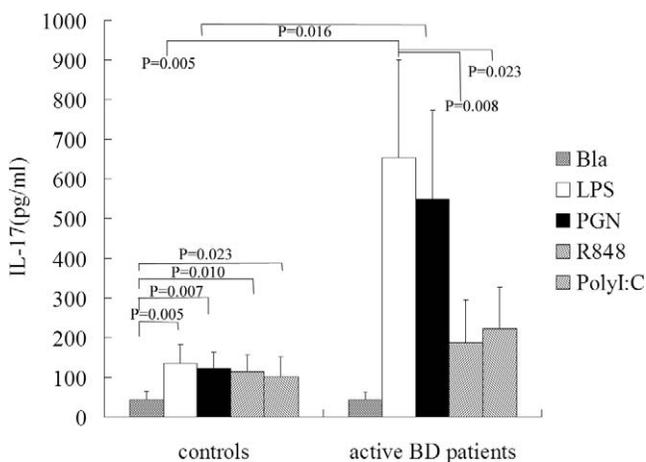
It is now becoming clear that both the immune response and infection are involved in the pathogenesis of BD.[27–30] How infection initiates the development of BD is not yet known and the studies described here hope to provide further insight into this matter. Our results showed a significantly higher expression of TLR2, TLR3, TLR4, and TLR8, which recognize PGN, PolyI:C, LPS, and R848, respectively, in BD patients as compared with controls. Our results confirm earlier observations describing increased levels of TLR2 and TLR4 in monocytes from active BD patients.[31] Our data are also in agreement with a Japanese study showing that upregulated TLR4 is associated with heme oxygenase-1 reduction in PBMCs from patients with BD.[32] The earlier studies in BD investigated the expression of one or two TLRs and our study is the first to study four TLRs at the same time. Although 10 TLRs have been identified in humans, we confined our study to the four TLRs that are best known at the present time. Future studies should be carried out to investigate the role of the other TLRs in BD. How TLRs exactly influence the pathogenesis of BD remains unknown. Whether an intrinsic enhanced TLR expression in BD makes these patients more susceptible to microbial ligands or whether ulceration induces an enhanced TLR expression,

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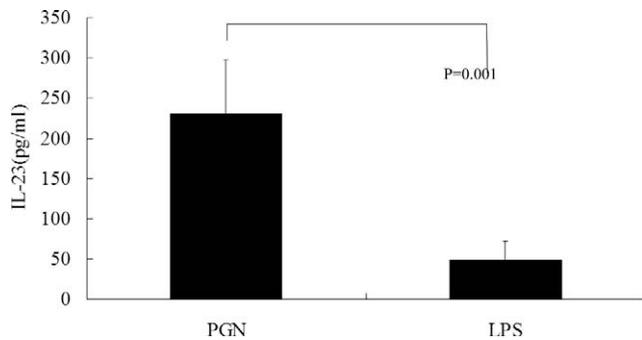
FIGURE 4. IL-23 production by monocytes stimulated with LPS or PGN from active BD patients ($n = 8$). Paired-samples t-test was applied for statistical analysis. Data are expressed as mean ± SD.

thereby starting a self-perpetuating inflammatory response needs to be clarified in the future. We observed a significantly higher expression of IL-1β and IL-23 by monocytes from BD patients stimulated with LPS and PGN as compared with the other two ligands. Increased TLRs have also been reported in other autoimmune diseases. Myles and Aggarwal,[33] for instance, found a higher expression of TLR2 and TLR4 in PBMCs and synovial fluid monocytes in patients with juvenile idiopathic arthritis. Midgley et al.[34] observed that the expression of TLR3, TLR8, TLR7, and TLR9 was higher in PBMCs from patients with juvenile systemic lupus erythematosus compared with controls.

Recent studies have shown that IL-1β and IL-23 are important cytokines capable of inducing the differentiation and proliferation of Th17 cells.[19,35] Th17 cells have been shown to be involved in the pathogenesis of BD.[21] In this study, we found a higher expression of TLR2 and TLR4, and higher levels of IL-1 and IL-23 when cells were stimulated with ligands of TLR2 and TLR4. Correspondingly, markedly higher levels of IL-17 were produced by naïve T cells when cocultured with monocytes stimulated with LPS and PGN in patients with BD as compared with controls. TLRs may act as a bridge between infection and autoimmunity by inducing IL-1β and IL-23, which

may subsequently play a role in the regulation of IL-17 production.

Both LPS and PGN induced the production of IL-17, IL-1β, and IL-23. No significant difference was observed with regard to the stimulatory effect on IL-17 and IL-1β production between LPS and PGN. However, a stronger effect on IL-23 production was observed when cells were stimulated with PGN as compared with LPS. These results suggest that LPS and PGN may exert their roles through the stimulation of different cytokines, although both are involved in the pathogenesis of BD. Whether our findings are unique for ocular BD is not yet clear, and further studies are needed to address TLR expression in other uveitis entities. Furthermore, it would be interesting to investigate whether the TLR response in BD changes in response to immunosuppressive treatment and if this response is lower during the remission phase of BD

In conclusion, we found a higher expression of TLR2, TLR3, TLR4, and TLR8 in active BD patients. Higher levels of IL-1β and IL-23 were produced in BD patients when stimulated with LPS and PGN. Higher levels of IL-17 were produced by cocultured naive T cells and monocytes following stimulation with LPS and PGN. These results suggest that an increased expression of TLRs may be involved in the pathogenesis of BD.

FIGURE 5. IL-17 production by naïve T cells cocultured with monocytes stimulated by LPS, PGN, R848, or PolyI:C from active BD patients ($n = 8$) and controls ($n = 8$). A *dark triangle* means that the levels of IL-17 in the groups stimulated with LPS, PGN, R848, or PolyI:C were significantly higher as compared to the unstimulated control ($P < 0.001$). ANOVA was used for statistical analyses. Data are expressed as mean ± SD.

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