IL-1β Triggered by Peptidoglycan and Lipopolysaccharide through TLR2/4 and ROS-NLRP3 Inflammasome–Dependent Pathways Is Involved in Ocular Behçet's Disease

Liang Liang,[1] *Xiaoyu Tan,*[1] *Qingyun Zhou,*[1] *Yi Zhu,*[1] *Yuan Tian,*[1] *Hongsong Yu,*[1] *Aize Kijlstra,*[2] *and Peizeng Yang*[1]

Purpose. Behçet's disease (BD) is a chronic systemic inflammatory disorder of unknown etiology. Toll-like receptors (TLRs) are critical in the innate immune response to microbial invaders. In this study we investigated the role of TLRs in the pathogenesis of BD.

Methods. TLR2/4 expression and IL-1β and reactive oxygen species (ROS) production were studied in monocyte-derived macrophages (MDMs) obtained from BD patients, acute anterior uveitis (AAU) patients, and healthy controls using real-time PCR, flow cytometry, and ELISA. The NLRP3 inflammasome of MDMs was downregulated by RNA interference. The levels of phosphorylated P38, Erk1/2, and JNK MAPK were evaluated using flow cytometry.

Results. TLR2/4 expression was significantly increased in MDMs from active BD patients. IL-1β and ROS production of peptidoglycan (PGN)/lipopolysaccharide (LPS)–induced MDMs from active BD patients was significantly increased compared with inactive BD patients, AAU patients, and healthy controls. ROS activator and inhibitor significantly increased and decreased the production of IL-1β, respectively. The production of IL-1β was significantly decreased after the NLRP3 inflammasome was downregulated. The phosphorylation levels of p38 and ERK1/2 in MDMs from BD patients and controls were increased following stimulation with either PGN or LPS. Both SB203580 (p38 inhibitor) and PD98059 (ERK1/2 inhibitor) significantly decreased the production of IL-1β.

Conclusions. The results suggest that TLR2/4 expression in MDMs from active BD patients is significantly increased. Interaction of TLR2/4 with their ligands PGN/LPS is involved in BD pathogenesis, possibly by the induction of IL-1β through a ROS-NLRP3–dependent pathway. (*Invest Ophthalmol Vis Sci.* 2013;54:402–414) DOI:10.1167/iovs.12-11047

Behçet's disease (BD) is characterized by recurrent uveitis, oral aphthae, genital ulcers, and skin lesions.[1,2] It is more prevalent in the region that extends from China and Japan in the Far East to the Mediterranean Sea.[2,3] The etiopathogenesis of Behçet's disease is still not completely understood. BD is currently seen as a disease at the crossroad between autoimmune and autoinflammatory syndromes, possibly triggered by an aberrant response to infectious stimuli.[4] *Staphylococcus aureus* and *Streptococcus oralis* have been identified in the skin lesions of BD patients[5,6] and Ayaslioglu et al.[7] found a higher IgA seropositivity to *Chlamydophila pneumoniae* in BD patients. Turkish studies indicate that an improved oral hygiene is associated with a decreased incidence of BD over the last decades.[8] These findings support a role for microorganisms in the development of BD, although their exact role remains unclear.

The innate immune system plays a crucial role in the inflammatory response to infection through the activity of receptors capable of recognizing defined molecular patterns present in a variety of microorganisms. Toll-like receptors (TLRs) are crucial players in the innate immune response to microbial invaders, enabling vertebrates to detect the pathogen-associated molecular patterns (PAMPs) early and subsequently activating the adaptive immune response.[9] However, the extensive release of TLR-triggered proinflammatory mediators may harm the host, as in cases of sepsis or chronic inflammatory disease.[10] Among 11 members of the TLR family, TLR2 and TLR4 have been identified as signaling receptors activated by bacterial wall components, such as peptidoglycan (PGN) from Gram-positive bacteria and lipopolysaccharide (LPS) from Gram-negative bacteria. Endogenous molecules such as HSP60 and fragmentation products of fibronectin can also trigger an inflammatory response via TLR2 and TLR4.[11] It has been shown that TLR2 and TLR4 are involved in BD pathogenesis.[12,13] Vitamin D3 has been shown to improve BD-like symptoms by downregulating the expression of TLR2/4 and proinflammatory cytokines in herpes simplex virus–induced Behçet's disease-like inflammation in a mouse model.[14] It has also been reported that gene expression of

From the [1]The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, and Chongqing Eye Institute, Chongqing, People's Republic of China; and the [2]University Eye Clinic Maastricht, Maastricht, The Netherlands.

Supported in part by Natural Science Foundation Major International (Regional) Joint Research Project Grant 30910103912, National Basic Research Program of China (973 Program) Grant 2011CB510200, Key Project of Natural Science Foundation Grant 81130019, National Natural Science Foundation Project Grant 30973242, Research Fund for the Doctoral Program of Higher Education of China Grant 20115503110002, Chongqing Key Laboratory of Ophthalmology (CSTC) Grant 2008CA5003, the Project of Medical Science and Technology of Chongqing, Program for the Training of a Hundred Outstanding S&T Leaders of Chongqing Municipality, and Fund for PAR-EU Scholars Program.

Submitted for publication September 28, 2012; revised November 1, 2012; accepted November 28, 2012.

Disclosure: **L. Liang**, None; **X. Tan**, None; **Q. Zhou**, None; **Y. Zhu**, None; **Y. Tian**, None; **H. Yu**, None; **A. Kijlstra**, None; **P. Yang**, None

Corresponding author: Peizeng Yang, The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, and Chongqing Eye Institute, Chongqing, P.R. China, 400016; peizengycmu@126.com.

Table 1. The Clinical Characteristics of Patients with Active BD

Patient Number	Age y	Sex	Extraocular Findings			
			Oral Ulcer	Genital Ulcer	Skin Lesions	Arthritis
1	26	M	+	+	+	+
2	32	M	+	-	+	+
3	41	F	+	+	+	+
4	25	F	+	+	+	-
5	33	M	+	+	+	-
6	21	F	+	+	+	+
7	34	F	+	-	+	-
8	32	M	+	-	+	-
9	45	M	+	+	+	+
10	26	M	+	-	+	-
11	32	F	+	+	+	-
12	28	M	+	+	+	-
13	37	F	+	+	+	+
14	25	F	+	-	+	-
15	31	M	+	-	+	-
16	27	M	+	+	+	+
17	32	F	+	-	+	-
18	36	F	+	+	+	-
19	23	M	+	+	+	-
20	47	M	+	+	+	+
21	23	F	+	-	+	-
22	45	F	+	-	+	-
23	36	M	+	+	+	+
24	22	F	+	+	+	-
25	41	M	+	+	+	-

Table 2. The Clinical Characteristics of Patients with Inactive BD

Patient Number	Age y	Sex	Extraocular Findings			
			Oral Ulcer	Genital Ulcer	Skin Lesions	Arthritis
1	34	F	+	+	-	-
2	23	M	+	-	+	-
3	36	F	+	+	-	-
4	27	M	+	+	+	+
5	45	F	+	+	+	-
6	28	F	+	-	+	-
7	36	M	+	+	+	+
8	41	F	+	-	+	-
9	23	M	+	+	+	-
10	35	M	+	+	-	-
11	22	F	+	+	-	+
12	37	M	+	-	+	-
13	48	M	+	+	+	-
14	32	M	+	-	+	-
15	41	M	+	-	+	-
16	32	F	+	+	-	-
17	36	M	+	-	+	-
18	43	M	+	+	+	+
19	25	F	+	+	-	-
20	48	M	+	+	-	-

TLR2 was downregulated in BD patients after starting infliximab treatment.[15] All these data suggest that TLR2/4 play a role in the development of BD. However, the exact mechanisms involved in the initiation of an innate immune response following the interaction of TLRs with their ligands are not clear and, therefore, constitute the subject of the study presented here. Our results showed that interaction of TLR2/4 with their ligands could significantly increase the ROS production of mitochondria and could activate the NLRP3 inflammasome to induce IL-1β production by monocyte-derived macrophages (MDMs).

Methods

Subjects

Twenty-five active BD patients (13 males and 12 females, with an average age of 32 years) and 20 inactive BD patients (12 males and 8 females, with an average age of 34.6 years) were included in this study. The clinical characteristics of the patients are shown in Tables 1 and 2. Twenty acute anterior uveitis (AAU) patients (12 males and 8 females, with an average age of 35.4 years) and 25 healthy individuals (12 males and 13 females, with an average age of 33.2 years) acted as control groups. All of these patients and controls were enrolled between April 2011 and June 2012. The diagnosis of Behçet's disease was based on the diagnostic criteria designed by the International Study Group for Behçet's Disease.[16] The active BD patients were selected in this study mainly based on an active intraocular inflammation rather than visual impairment because it could be influenced by a number of complications such as vitreous opacities, complicated cataract, secondary glaucoma, occlusion of retinal blood vessels, atrophy of the optic nerve, and retinal atrophy. The active ocular BD patients included in this study showed active intraocular inflammation, as evidenced by decreased vision (100%), dust keratic precipitates (100%), flare and cells in the anterior chamber (100%), vitreous cells (84%), and retinal vasculitis (100%) detected by fundus fluorescein angiography. The 25 BD patients with active uveitis enrolled in this study were all on their first visit in our hospital. Twelve did not use any immunosuppressive agents either due to not being referred to a hospital or due to the worry about the side effects of systemic corticosteroids. In the other 13 patients, only a low dose of systemic corticosteroids (<20 mg/d) was used within the past 2 months, but patients had stopped using the drugs for some days before visiting us and sampling. We normally treat BD patients using systemic corticosteroids in combination with cyclosporine, cyclophosphamide, or chlorambucil for >1.5 years. The drug dose was gradually tapered after the intraocular inflammation was controlled and the treatment usually stopped 6 months after complete control of the intraocular inflammation. After termination of all medications for at least 2 months we collected the blood sample from these inactive BD patients. Written and informed consent was obtained from all patients and healthy controls. All procedures met the tenets of the Declaration of Helsinki and were approved by our Clinical Ethical Research Committee.

Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood by density-gradient centrifugation (Ficoll-Hypaque Density Media; Sigma-Aldrich, St. Louis, MO). Monocytes were purified from PBMCs by using human CD14 microbeads according to the manufacturer's instructions (Miltenyi Biotec, Palo Alto, CA). Monocytes were resuspended in RPMI medium 1640 (RPMI 1640 Medium; Invitrogen, Carlsbad, CA) containing penicillin–streptomycin (1%), glutamine (1%), heat-inactivated fetal bovine serum (FBS) (10%), and heat-inactivated normal hamster serum (5%) (complete medium), and seeded into 24-well plastic plates at a concentration of 1.0×10^6 cells/well and were cultivated for 7 to 8 additional days at 37°C in 5% CO_2 to promote their full differentiation into MDMs.[17] The MDMs were incubated for 4 hours with or without LPS (100 ng/mL; Sigma-Aldrich) or PGN (5 μg/mL; Fluka Chemie GmbH, Buchs, Switzerland). TLR-blocking experiments were performed using 20 μg/mL anti-TLR2 (clone TL2.1) and 20 μg/mL anti-TLR4 (clone HTA125) antibodies (functional grade, endotoxin-tested; eBioscience, San

Diego, CA) for 1 hour prior to the addition of PGN or LPS. The p38 inhibitor SB203580 and ERK1/2 inhibitor PD98059 (10 μM; Sigma-Aldrich) were added to cultures 30 minutes before PGN/LPS stimulation. The following inhibitors were obtained from Sigma-Aldrich and used at the final concentrations: rotenone (10 μM) and diphenyleneiodonium chloride (DPI) (5 μM).

Flow Cytometric Analyses

Mitochondrial mass was measured by fluorescence levels upon staining (Mitotracker green and Mitotracker deep red; Invitrogen) at 50 nM for 30 minutes at 37°C. Mitochondria-associated ROS levels were measured by staining cells with a commercial stain (MitoSOX; Invitrogen) at 2.5 mM for 30 minutes at 37°C. Cells were then washed with PBS solution and resuspended in cold PBS solution containing 1% FBS for fluorescence-activated cell sorting (FACS) analysis.

To investigate the expression of TLR2 and TLR4, MDMs were incubated for 30 minutes at 4°C with anti-human CD282 (TLR2)/CD284 (TLR4) PE (eBioscience) and mouse IgG2a K Isotype Control PE (eBioscience). Cells were then washed with PBS solution and resuspended in cold PBS solution for FACS analysis.

Phosphospecific flow cytometry[18] was used in the present study to detect the JNK, ERK, and p38 protein phosphorylation levels. At the end of the cell treatments, the cells were rapidly detached and fixed immediately by adding 250 μL prewarmed fixation buffer (eBioscience). The cells were then incubated at 37°C for 15 minutes followed by washing twice with cold PBS by centrifugation at 500*g* for 5 minutes. Immediately after washing, the cells were permeabilized by adding cold permeabilization buffer (eBioscience) and incubated for a minimum of 30 minutes at 4°C. Finally, the cells were washed twice with PBS for 5 minutes by centrifugation at 500*g* and resuspended in PBS at a concentration of 1×10^6 cells/mL. To each tube were added 20 μL anti-phospho-ERK1/2, 20 μL anti-phospho-p38, and 5 μL anti-phospho-JNK antibody (BD Bioscience, San Diego, CA). Flow cytometry was conducted on FACS Aria, and the data were analyzed using commercial statistical software (FACSDiva Software; BD Bioscience).

ELISA for IL-1β

The IL-1β level in the supernatants was measured with a commercial ELISA kit (Human DuoSet ELISA Development Kit; R&D Systems, Minneapolis, MN) according to the manufacturer's protocols.

Real-Time Quantitative PCR Analysis

Total RNA was extracted with a commercial reagent (TRIzol; Invitrogen) following the manufacturer's instructions. RNA concentrations were determined with a commercial nano instrument (NanoDrop Technologies, Wilmington, DE). The first-strand cDNA was synthesized for each RNA sample using a commercial system (Superscript III Reverse Transcriptase System; Invitrogen). Real-time quantitative PCR was performed on a commercial PCR detection system (iCycler; Bio-Rad Laboratories Ltd., Hertfordshire, UK) using a commercial kit (Quanti Tect SYBR Green PCR Kit; Applied Biosystems, Foster City, CA). The forward and reverse primers for β-actin were designed using commercial software (Primer Premier Software; PREMIER Biosoft International, Palo Alto, CA) as follows: β-actin forward, 5′-GGA TGC AGA AGG AGA TCA CTG-3′ and β-actin reverse, 5′-CGA TCC ACA CGG AGT ACT TG-3′. The RNA of TLR2/4 was reverse transcribed and amplified with the following primers (Qiagen, Valencia, CA): TLR2 forward, 5′-GGA GGC TGC ATA TTC CAA GG-3′ and TLR2 reverse, 5′-GCC AGG CAT CCT CAC AGG-3′; TLR4 forward, 5′-AGT TTC CTG CAA TGG ATC AAGG-3′ and TLR4 reverse, 5′-CTG CTT ATC TGA AGG TGT TGC AC-3′. For each sample, the relative abundance of target mRNA was calculated from the obtained $C_{\Delta t}$ values for both target and endogenous reference gene β-actin by applying the following formula: relative mRNA expression = $2[C_{\Delta t}(\beta\text{-actin}) - C_{\Delta t}(\text{target})]$.

Lentivirus-Mediated Inhibition of NLRP3

The NLRP3-RNAi-LV and pGC-FU-RNAi-NC-LV as controls were purchased commercially (GeneChem, Shanghai, China). MDMs were plated on 96-well plates at a density of 2×10^5 cells/mL, and infected according to the user's manual.

Statistical Analysis

Student's *t*-test and one-way ANOVA were applied using commercial software (SPSS17.0; SPSS Inc., Chicago, IL). Data are shown as mean ± SD. Values of $P < 0.05$ were considered significant.

Results

Increased Expression of TLR2 and TLR4 in MDMs from Patients with Active BD

Real-time quantitative PCR analysis was performed to quantify the expression of TLR2 and TLR4 transcripts among BD patients, AAU patients, and healthy controls. The results showed that the levels of TLR2 and TLR4 mRNA in MDMs were significantly increased in active BD patients as compared with inactive BD, AAU patients, and healthy controls (Figs. 1A, 1B). A similar result was also observed in the cell surface expression of TLR2/4 by flow cytometry (Figs. 1C–F). There was no difference concerning the RT-PCR results and flow cytometric analysis results among inactive BD patients, AAU controls, and healthy controls.

PGN and LPS Stimulate the Production of IL-1β by MDMs

The increased expression of TLR2/4 in MDMs of active BD patients raises a possibility that both TLRs could be involved in this disease through interaction with their ligands, PGN and LPS. Further experiments were therefore performed to investigate the effects of their interaction on the production of IL-1β, an important cytokine already proven to be involved in IL-17 production and BD pathogenesis.[19,20] The results showed that the IL-1β production by MDMs in active BD patients was significantly higher than that in inactive BD, AAU patients, and healthy controls. Both PGN and LPS significantly increased the production of IL-1β by MDMs in all tested groups. However, the production of IL-1β by MDMs in active BD patients was significantly higher than that in inactive BD, AAU patients, and healthy controls after stimulation with PGN or LPS. There was no difference between inactive BD patients, AAU patients, or healthy controls concerning the production of IL-1β with or without PGN/LPS stimulation. Addition of anti-TLR2 and anti-TLR4 antibodies to the cultures showed that both antibodies could significantly decrease the production of IL-1β by PGN/LPS-stimulated MDMs in BD patients, AAU patients, and healthy controls (Figs. 2A, 2B).

ROS from Mitochondria Are Involved in the Production of IL-1β by PGN/LPS-Stimulated MDMs

It has been reported that IL-1β production by macrophages could be induced by mitochondrial ROS.[21] Further experiments were performed to investigate whether the increased IL-1β by MDMs was due to mitochondrial dysfunction and increased ROS production induced by the interaction of TLR2/4 with their ligands. We first investigated the levels of mitochondrial dysfunction and ROS production in BD patients, AAU patients, and healthy controls. The result showed that the

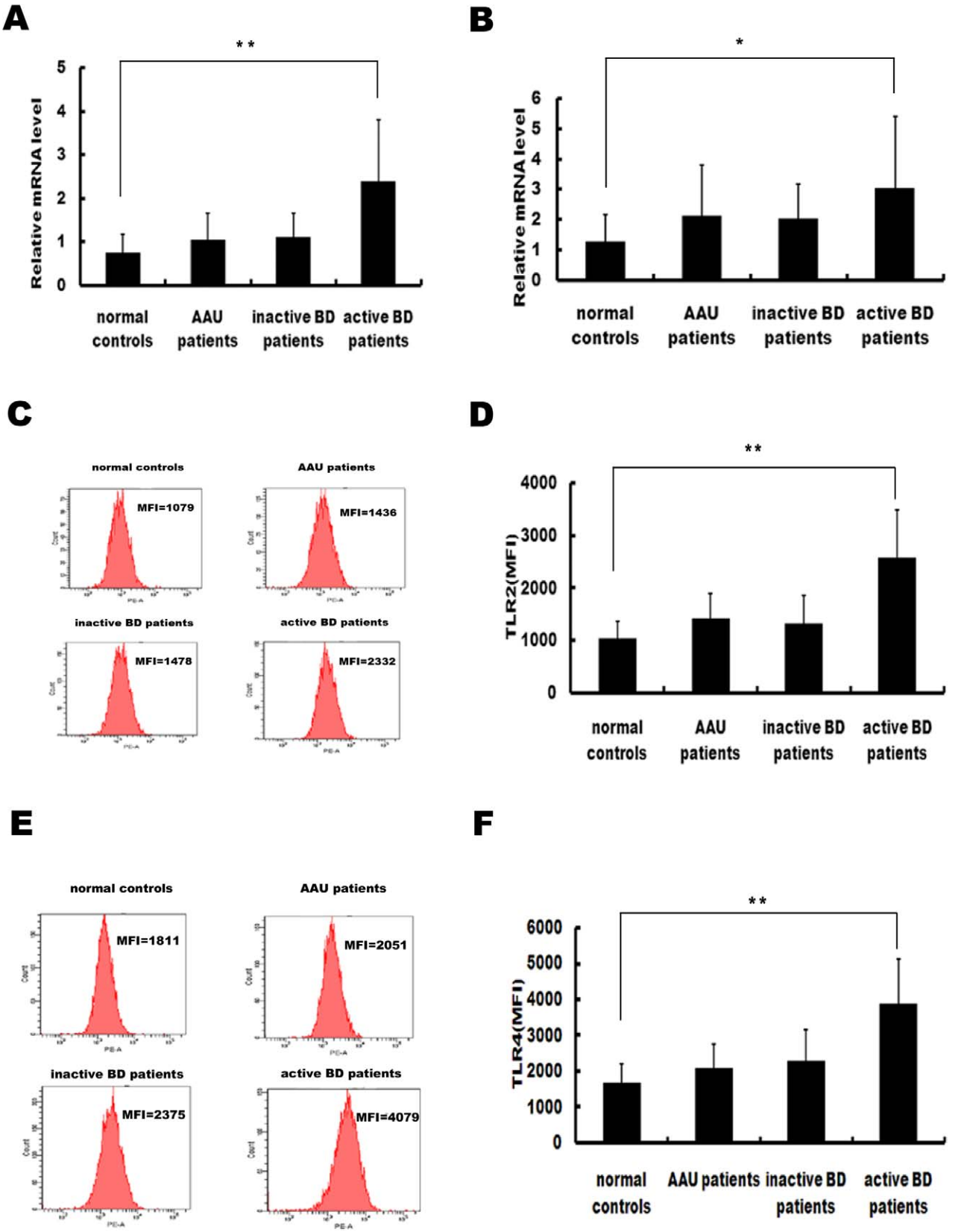


Figure 1. Increased expression of TLR2, TLR4 on MDMs from BD patients with active uveitis. MDMs from normal controls ($n = 10$), AAU patients ($n = 10$), inactive BD patients ($n = 10$), and active BD patients ($n = 10$) were assessed. (**A**) Real-time PCR analysis of TLR2 mRNA expression. (**B**) Real-time PCR analysis of TLR4 mRNA expression. (**C**, **D**) Flow cytometric analysis of TLR2 protein expression. (**E**, **F**) Flow cytometric analysis of TLR4 protein expression. $^{*}P < 0.05$, $^{**}P < 0.01$.

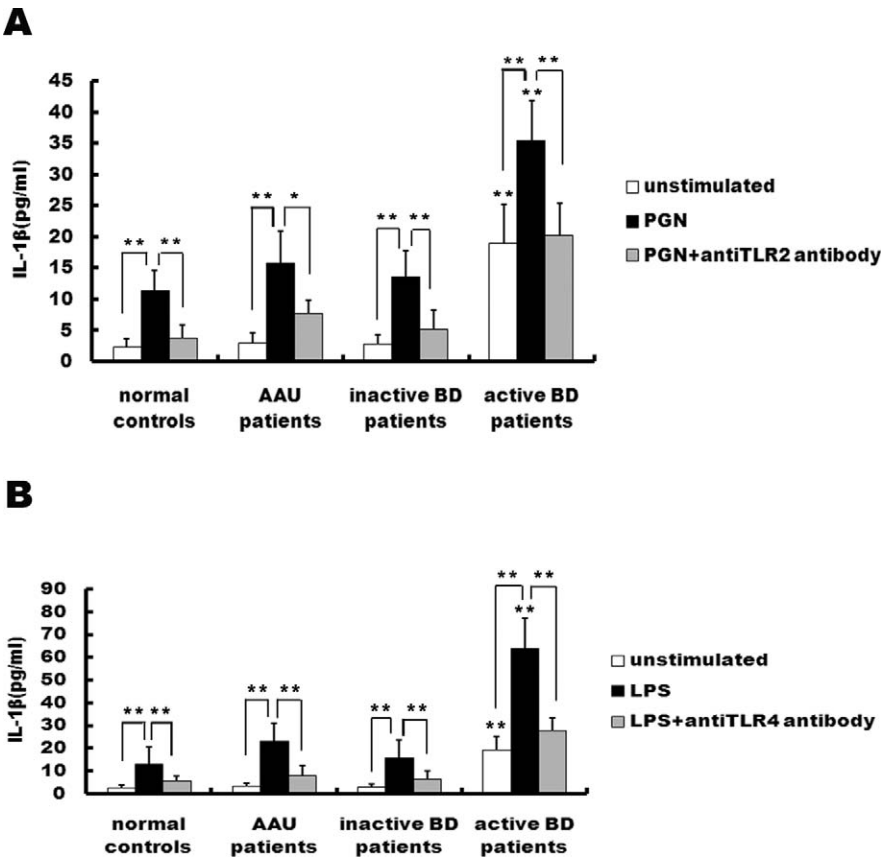


Figure 2. PGN and LPS stimulate the production of IL-1β by MDMs. MDMs from normal controls ($n = 10$), AAU patients ($n = 10$), inactive BD patients ($n = 10$), and active BD patients ($n = 10$) were assessed. (**A**) MDMs were stimulated with PGN in the presence or absence of anti-TLR2 antibody for 4 hours. IL-1β in the supernatants was analyzed by ELISA. (**B**) MDMs were stimulated with LPS in the presence or absence of anti-TLR4 antibody for 4 hours. IL-1β in the supernatants was analyzed by ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$.

percentage of dysfunctional mitochondria and ROS production of MDMs in active BD patients was significantly higher than that in inactive BD, AAU patients, and healthy controls. PGN and LPS could significantly increase the percentage of dysfunctional mitochondria and production of ROS of MDMs from all tested groups. The percentage of dysfunctional mitochondria and production of ROS in active BD patients was significantly higher than that of inactive BD, AAU patients, and healthy controls after stimulation with PGN or LPS. There was no difference among inactive BD patients, AAU patients, and healthy controls in the percentage of dysfunctional mitochondria and ROS production of MDMs with or without PGN/LPS stimulation (Figs. 3A, 3B). Further experiments using anti-TLR2 and anti-TLR4 antibodies showed that both antibodies could significantly decrease the percentage of dysfunctional mitochondria and the production of ROS by PGN/LPS-induced MDMs in BD patients, AAU patients, and healthy controls (Figs. 3A, 3B).

We subsequently performed experiments to examine the effect of mitochondrial dysfunction-driven ROS induced by PGN/LPS on the production of IL-1β. The result showed that rotenone and DPI, which promoted or inhibited mitochondrial ROS generation,[22,23] also significantly promoted or inhibited the production of IL-1β by PGN/LPS-induced MDMs from all groups (Fig. 3C). These data strongly suggested that ROS from mitochondria are involved in PGN/LPS-induced IL-1β production by MDMs.

NLRP3 Inflammasome Is Involved in the Induction of ROS on IL-1β Production by PGN/LPS-Stimulated MDMs

It has been reported that the NLRP3 inflammasome is involved in the production of IL-1β.[24] A further experiment with RNA interference was performed to investigate whether the aforementioned induction of ROS on IL-1β production was mediated through NLRP3. Our results showed that NLRP3 small interfering RNA (siRNA) successfully reduced the expression of NLRP3 in MDMs by >70% from all tested groups (Figs. 4A, 4B). Further experiments showed that the production of IL-1β by PGN/LPS-stimulated MDMs from BD patients, AAU patients, and healthy controls was significantly decreased after NLRP3 was downregulated (Fig. 4C). Collectively, these results show that the NLRP3 inflammasome is involved in the induction of ROS on IL-1β production by PGN/LPS-induced MDMs.

ROS Activates the NLRP3 Inflammasome through P38 and ERK1/2 Activation

The aforementioned result revealed the involvement of the NLRP3 inflammasome in the production of IL-1β. We further examined whether ROS exerted its effect on the NLRP3 inflammasome in the tested patients and healthy controls through the activation of mitogen-activated protein kinases (MAPK). The result showed that upregulated phosphoryla-

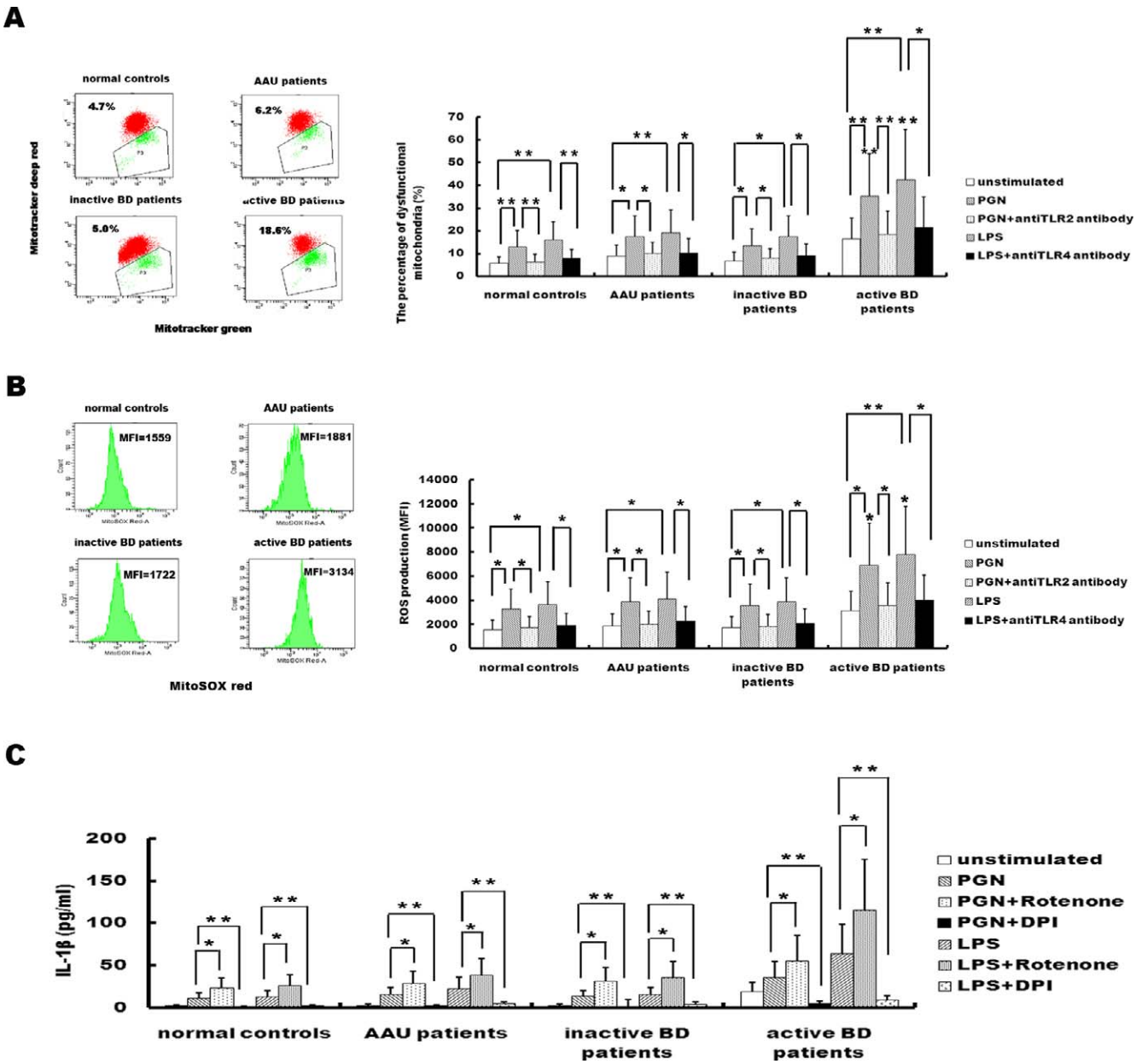


Figure 3. ROS from mitochondria are involved in PGN/LPS-induced IL-1β production by MDMs. MDMs from normal controls ($n = 8$), AAU patients ($n = 8$), inactive BD patients ($n = 8$), and active BD patients ($n = 8$) were stimulated with PGN or LPS in the presence or absence of anti-TLR2 antibody or anti-TLR4 antibody for 4 hours. (**A**) Flow cytometric analysis of dysfunctional mitochondria in MDMs. (**B**) Flow cytometric analysis of ROS production in MDMs. (**C**) ELISA of IL-1β production in the supernatants of MDMs stimulated with PGN or LPS in the presence or absence of rotenone or DPI. $^{*}P < 0.05$, $^{**}P < 0.01$.

tion of P38 and Erk1/2 was observed in BD patients, AAU patients, and healthy controls following stimulation with PGN or LPS, although the levels of phosphorylated P38 and Erk1/2 of MDMs were significantly higher in active BD patients as compared with inactive BD patients, AAU patients, and healthy controls. There was no difference concerning JNK phosphorylation among the test groups with or without PGN/LPS stimulation (Figs. 5, 6). A further experiment was performed to explore whether PGN/LPS activated P38 and Erk1/2 phosphorylation in MDMs was mediated by ROS. An experiment with DPI, an inhibitor for ROS, showed that it could significantly downregulate the phosphorylation of P38 and Erk1/2 in MDMs from all groups (Fig. 7). A further experiment was performed to examine whether the inhibitors for both P38 and Erk1/2 could block the effect of ROS on the IL-1β production. The result showed that SB203580 (P38 inhibitor) or PD98059 (ERK1/2 inhibitor) significantly inhibited the production of IL-1β, respectively. The combination of these two inhibitors led to a stronger inhibitory effect on IL-1β production (Fig. 8).

Discussion

In the present study, we showed a significantly increased expression of TLR2 and TLR4 in MDMs from patients with

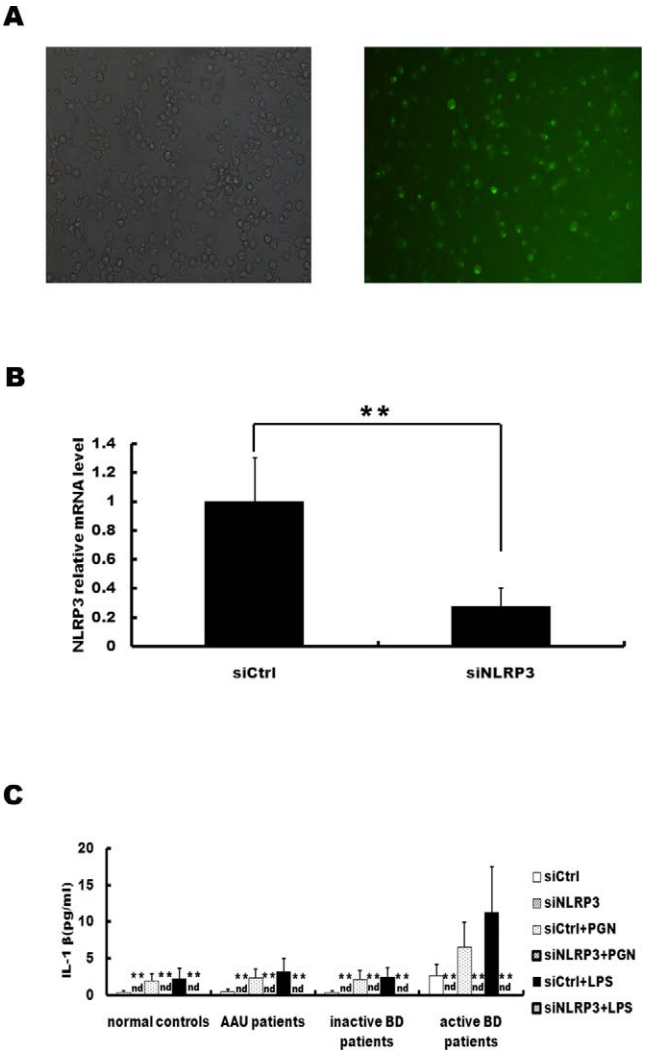


FIGURE 4. NLRP3 inflammasome is involved in the induction of ROS on IL-1β production by PGN/LPS-stimulated MDMs. (**A**) Human MDMs transfected with GFP-labeled pGC-FU-RNAi-NC-LV were observed by inverted fluorescent microscopy. (**B**) Real-time PCR analysis of NLRP3 expression in the MDMs transfected with siRNA or control siRNA. (**C**) ELISA of IL-1β production in the supernatants of MDMs stimulated with PGN or LPS after NLRP3 was downregulated by siRNA ($n = 8$). nd, not detectable. $^{**}P < 0.01$.

active BD as compared with inactive BD, AAU patients, and healthy controls. A significantly higher level of IL-1β was observed in the supernatants of cultured MDMs with or without stimulation with PGN/LPS in active BD patients compared with inactive BD patients and controls. Furthermore, we found that ROS from mitochondria could activate the NLRP3 inflammasome and, in turn, resulted in IL-1β production by MDMs. Experiments with inhibitors showed that ROS activated the NLRP3 inflammasome through the activation of P38 and Erk1/2. These results collectively suggest that TLR2 and TLR4 may be involved in the pathogenesis of BD by upregulating the production of IL-1β via the ROS–NLRP3 inflammasome pathway.

Abundant evidence indicates that both infection and immune response are involved in the pathogenesis of Behçet's disease.[25–27] However, it is not clear how infection induces an immune response and initiates the development of Behçet's disease. TLR2 and TLR4 have been suggested to be involved in this disease.[12,13,15] In this study we tested whether an abnormal expression of both TLRs correlated with disease activity in BD patients. Our study showed that a higher expression of these two TLRs both in the mRNA level and the protein level was associated with BD activity. Furthermore, we found that the correlation of higher expression of both TLRs with disease activity was present only in BD, rather than in AAU. These results suggest that an increased expression of these two TLRs may be a unique phenomenon in active BD patients. Our result is, by and large, consistent with an earlier study by Do et al.[28] They found an increased TLR2 expression at both the mRNA level and the protein level and an increased TLR4 expression at the mRNA level in monocytes from active BD patients.

In view of the increased expression of TLR2 and TLR4 on MDMs in active BD patients, we further investigated whether upregulated TLR2 and TLR4 correlated with the expression of IL-1β, an important cytokine involved in the differentiation and proliferation of Th17 cells and BD pathogenesis.[19,20] Our results showed that an increased IL-1β level was present in the supernatants of cultured MDMs in active BD patients. This result identified an association of the increased expression of TLR2 and TLR4 with upregulated IL-1β production in active BD. Our further experiments showed that the interaction of TLR2 or TLR4 with their ligands, PGN and LPS, was able to induce IL-1β production by MDMs in all the tested groups. This result is consistent with those reported previously.[29,30] Wiken et al.[29] reported that the interaction of TLR2 and PGN was able to stimulate mononuclear cells from sarcoidosis patients to stimulate the production of IL-1β and other proinflammatory cytokines. Ren et al.[30] showed that interaction of TLR4 with LPS promoted the production of IL-1β, TNF-α, and IL-6 by alveolar macrophages through the MyD88 pathway and, in turn, lead to lung inflammation. The in vitro results mentioned earlier suggest that an upregulated IL-1β production induced by the interaction of TLR2 or TLR4 with their ligands may play a causative role in these diseases. This hypothesis would have been supported by an analysis of circulating IL-1β levels in the serum of active and inactive patients. Unfortunately, the levels of IL-1β were below the detection limits of the assay used. This is consistent with an earlier study.[31] Although the levels of IL-1β in the serum are not clear, a higher level production of IL-1β in the synovial fluid from BD patients has been reported.[32] Furthermore, a recent pilot study using XOMA 052 (gevokizumab), a recombinant humanized anti-interleukin 1β antibody, was shown to be effective and safe in the treatment of BD patients with uveitis.[20] All these data support our in vitro data and suggest that IL-1β is involved in the pathogenesis of BD and may offer a potential target for the treatment of this disease.

It has long been known that ROS are predominantly produced by mitochondria and play essential roles in the immune response to invading pathogens. They have been implicated in the pathogenesis of inflammatory diseases including rheumatoid arthritis, multiple sclerosis, and thyroiditis.[33–35] ROS are able to promote the production of a number of proinflammatory cytokines, including IL-1β.[21,36] In this study we investigated the effect of ROS from mitochondria on the production of IL-1β. The results showed a significantly higher level of ROS from mitochondria in patients with active BD patients as compared with inactive BD patients, AAU patients, and healthy controls. Additionally, a higher production of IL-1β in association with a higher ROS level was noted in active BD patients when exposed to PGN or LPS. The experiment with a ROS promoter or inhibitor further demonstrated the critical role of ROS in the induction of IL-1β induced by the interaction of TLR2/TLR4 and their ligands.

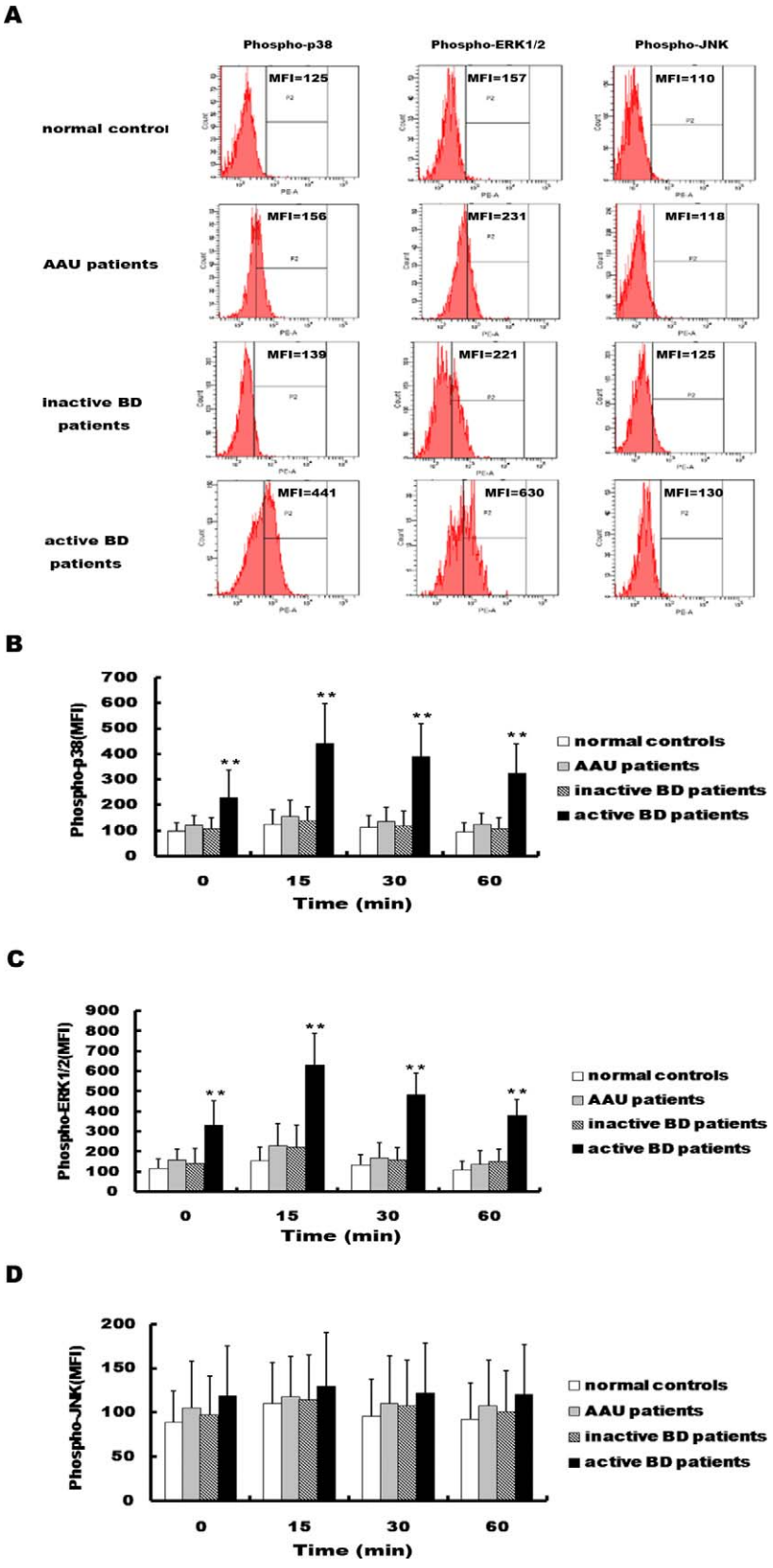


Figure 5. PGN induced P38 and ERK1/2 activation. MDMs from normal controls ($n = 8$), AAU patients ($n = 8$), inactive BD patients ($n = 8$), and active BD patients ($n = 8$) were stimulated with PGN (5 μg/mL) at the time points indicated. The phosphorylation of MAPK was determined by flow cytometry. (**A**) Representative histograms for phosphorylation of MAPK are shown at 15-minute post-PGN treatment. (**B**) MFI of phosphor-p38. (**C**) MFI of phosphor-ERK1/2. (**D**) MFI of phosphor-JNK. Data are expressed as mean ± SD. $^{**}P < 0.01$.

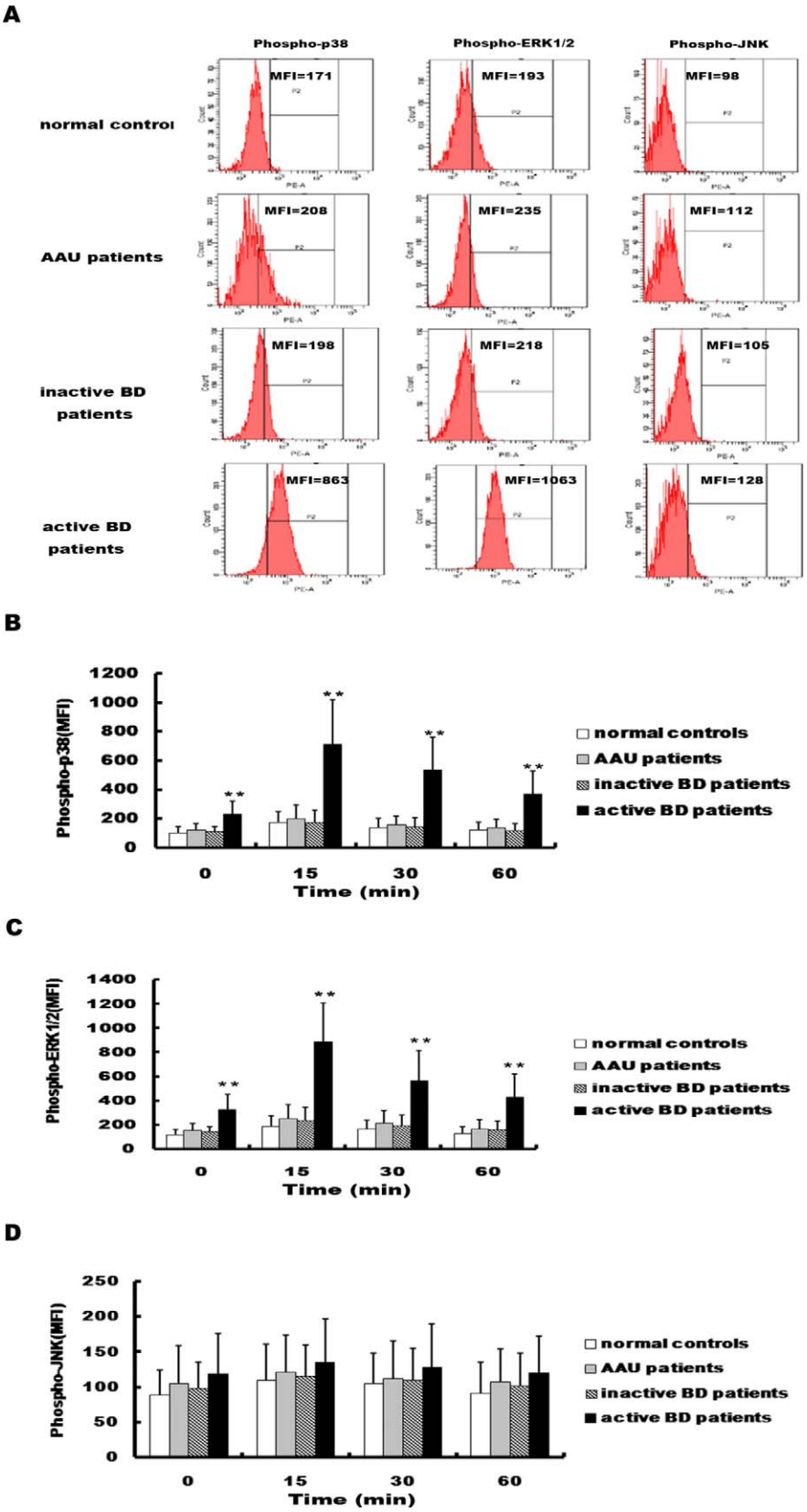
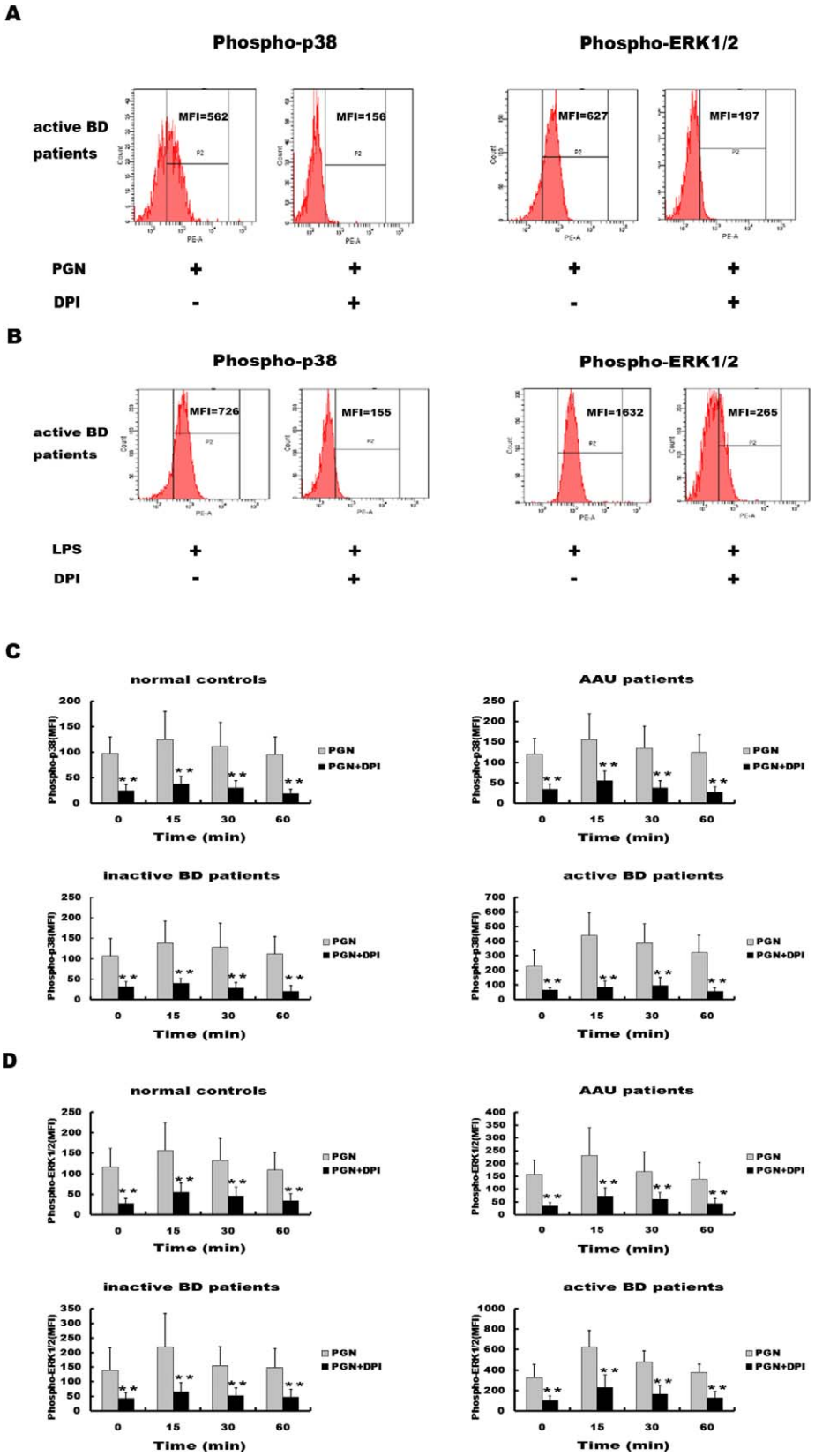


Figure 6. LPS induced P38 and ERK1/2 activation. MDMs from normal controls ($n = 8$), AAU patients ($n = 8$), inactive BD patients ($n = 8$), and active BD patients ($n = 8$) were stimulated with LPS (100 ng/mL) at the time points indicated. The phosphorylation of MAPK was determined by flow cytometry. (**A**) Representative histograms for phosphorylation of MAPK are shown at 15-minute post-LPS treatment. (**B**) MFI of phosphor-p38. (**C**) MFI of phosphor-ERK1/2. (**D**) MFI of phosphor-JNK. Data are expressed as mean ± SD. $^{**}P < 0.01$.



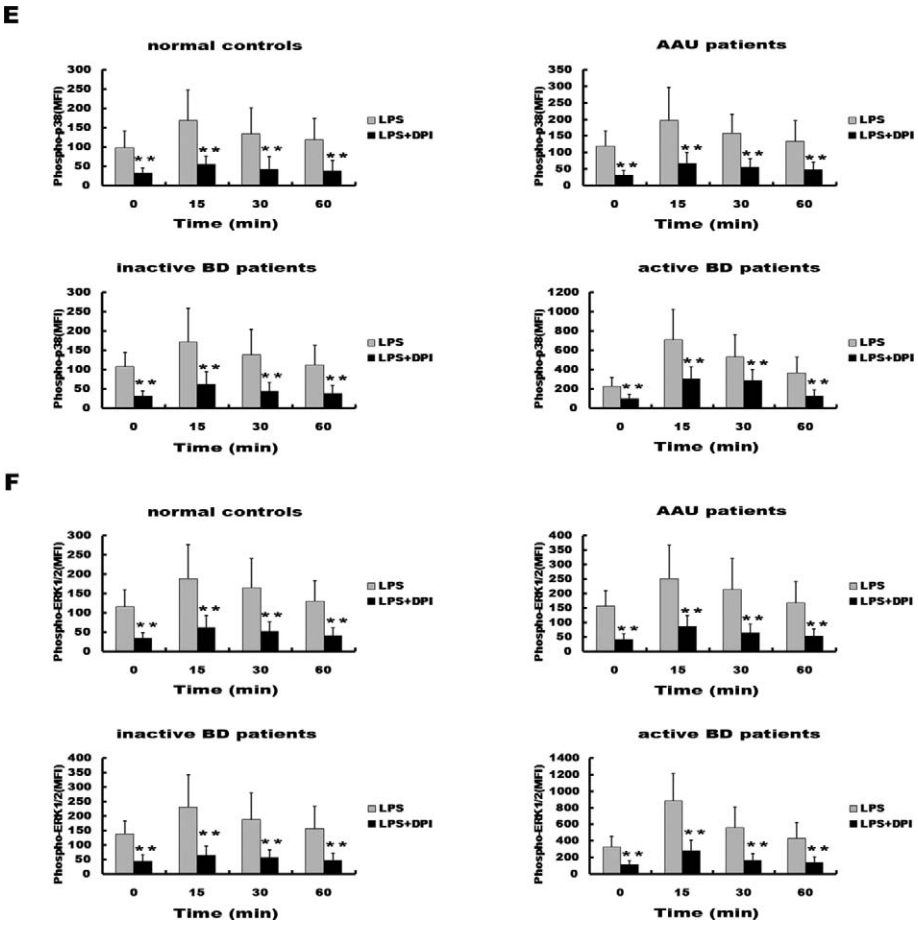


Figure 7. Effects of DPI on P38 and ERK1/2 activation. MDMs from normal controls ($n = 8$), AAU patients ($n = 8$), inactive BD patients ($n = 8$), and active BD patients ($n = 8$) were stimulated with PGN (5 μg/mL) or LPS (100 ng/mL) in the presence or absence of DPI (5 μM) at the time points indicated. (**A**) Representative histograms for phosphorylation of MAPK are shown at 15-minute post-PGN treatment. (**B**) Representative histograms for phosphorylation of MAPK are shown at 15-minute post-LPS treatment. (**C**) MFI of phosphor-p38 with PGN treatment. (**D**) MFI of phosphor-ERK1/2 with PGN treatment. (**E**) MFI of phosphor-p38 with LPS treatment. (**F**) MFI of phosphor-ERK1/2 with LPS treatment. Data are expressed as mean ± SD. $^{**}P < 0.01$.

In view of the aforementioned result, the question was raised as to how ROS promoted the production of IL-1β. The NLRP3 inflammasome has been shown to be a molecular platform activated upon signs of cellular "danger" to trigger innate immune defenses through the maturation of proinflammatory cytokines such as, for example, IL-1β.[24] Strong association of a number of human heritable and acquired diseases with dysregulated inflammasome activity highlights the importance of the NLRP inflammasome in regulating immune responses.[37] In this study we investigated whether ROS from mitochondria could stimulate the production of IL-1β through activation of the NLRP3 inflammasome. An experiment with RNA interference showed that a downregulated NLRP3 inflammasome expression was associated with decreased IL-1β production. Our observations are in agreement with those reported by Zhou and colleagues.[21] In an experiment using an NLRP3 inflammasome-knock out in the THP1 cell line, they failed to find the production of IL-1β upon stimulation with LPS. Importantly, for the first time, our study revealed that ROS was able to activate the NLRP3 inflammasome through the activation of P38, Erk1/2. Collectively, these results suggest that ROS from mitochondria promoted the production of IL-1β by MDMs through activation of the NLRP3 inflammasome.

A limitation of our study is that we studied only BD patients with ocular disease. BD is a systemic disease and there are BD patient groups lacking ocular manifestations. Further studies are needed to confirm whether our findings can be extrapolated to these other subgroups of BD. Another point of discussion is whether the use of steroids may have influenced our data. In our group of active BD patients, some patients had used a low dose of systemic corticosteroids (<20 mg/d) in the past 2 months. These patients had stopped using the drugs for some days before visiting our clinic and we therefore assumed that the effect of systemic corticosteroids on the immune system in these patients was small. Despite the fact that in some "active" patients corticosteroids may have affected our data, there were still clear differences between these patients and the "inactive" patients. Further studies are needed to clarify the effect of low-dose corticosteroid use on the assays performed in our study. In conclusion, our study revealed that interaction of TLR2/4 with their ligands triggered IL-1β secretion through ROS-NLRP3 inflammasome–dependent pathways is involved in the development of ocular BD, but not in AAU. These results establish a potential correlation between

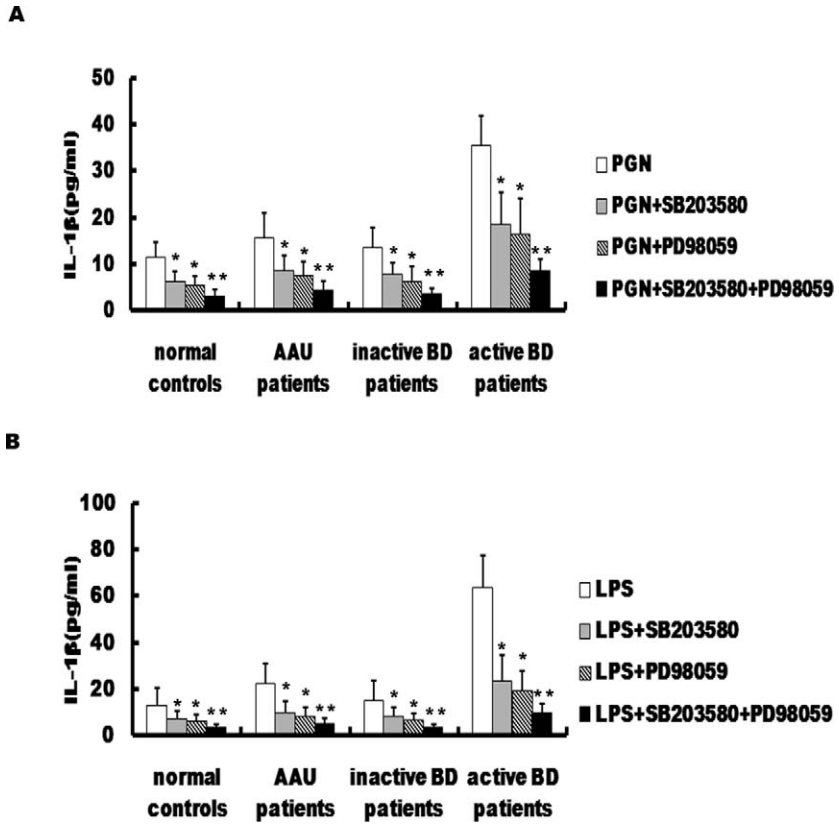


Figure 8. Effects of SB203580 or PD98059 on the production of IL-1 β. MDMs from normal controls ($n = 8$), AAU patients ($n = 8$), inactive BD patients ($n = 8$), and active BD patients ($n = 8$) were stimulated with PGN (5 μg/mL) or LPS (100 ng/mL) in the presence or absence of SB203580 (10 μM) or PD98059 (10 μM). (**A**) ELISA of IL-1β production in the supernatants of MDMs stimulated with PGN in the presence or absence of SB203580 or PD98059. (**B**) ELISA of IL-1β production in the supernatants of MDMs stimulated with LPS in the presence or absence of SB203580 or PD98059. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

bacterial infection and the development of an autoinflammatory disease. Furthermore, our study provides a support to the use of anti-IL-1β as a potential treatment for BD[20]; however, it is not known whether other TLRs may also be involved in BD pathogenesis. Our study investigated the activation of the NLRP3 inflammasome through ROS only from mitochondria. It is not clear whether activation of the inflammasome via other pathways including ATP-sensitive ion channels[38,39] is involved in BD pathogenesis and more studies are needed to address this issue.

References

1. Suzuki Kurokawa M, Suzuki N. Behçet's disease. *Clin Exp Med*. 2004;4:10–20.
2. Deuter CM, Kötter I, Wallace GR, Murray PI, Stübiger N, Zierhut M. Behçet's disease: ocular effects and treatment. *Prog Retin Eye Res*. 2008;27:111–136.
3. Sakane T, Takeno M, Suzuki N, Inaba G. Behçet's disease. *N Engl J Med*. 1999;341:1284–1291.
4. Pineton de Chambrun M, Wechsler B, Geri G, Cacoub P, Saadoun D. New insights into the pathogenesis of Behçet's disease. *Autoimmun Rev*. 2012;11:687–698.
5. Hatemi G, Bahar H, Uysal S, et al. The pustular skin lesions in Behcet's syndrome are not sterile. *Ann Rheum Dis*. 2004;63: 1450–1452.
6. Narikawa S, Suzuki Y, Takahashi M, Furukawa A, Sakane T, Mizushima Y. Streptococcus oralis previously identified as uncommon 'Streptococcus sanguis' in Behcet's disease. *Arch Oral Biol*. 1995;40:685–690.
7. Ayaşlioğlu E, Düzgün N, Erkek E, Inal A. Evidence of chronic Chlamydia pneumoniae infection in patients with Behçet's disease. *Scand J Infect Dis*. 2004;36:428–430.
8. Mumcu G, Ergun T, Inanc N, et al. Oral health is impaired in Behçet's disease and is associated with disease severity. *Rheumatology (Oxford)*. 2004;43:1028–1033.
9. Kaisho T, Akira S. Toll-like receptor function and signaling. *J Allergy Clin Immunol*. 2006;117:979–987; quiz 988.
10. Andreakos E, Foxwell B, Feldmann M. Is targeting Toll-like receptors and their signaling pathway a useful therapeutic approach to modulating cytokine-driven inflammation? *Immunol Rev*. 2004;202:250–265.
11. Vabulas RM, Ahmad-Nejad P, da Costa C, et al. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J Biol Chem*. 2001;276:31332–31339.
12. Nara K, Kurokawa MS, Chiba S, et al. Involvement of innate immunity in the pathogenesis of intestinal Behçet's disease. *Clin Exp Immunol*. 2008;152:245–251.
13. Kirino Y, Takeno M, Watanabe R, et al. Association of reduced heme oxygenase-1 with excessive Toll-like receptor 4 expression in peripheral blood mononuclear cells in Behçet's disease. *Arthritis Res Ther*. 2008;10:R16.
14. Do JE, Kwon SY, Park S, Lee ES. Effects of vitamin D on expression of Toll-like receptors of monocytes from patients with Behçet's disease. *Rheumatology (Oxford)*. 2008;47:840–848.
15. Keino H, Watanabe T, Taki W, Okada AA. Effect of infliximab on gene expression profiling in Behçet's disease. *Invest Ophthalmol Vis Sci*. 2011;52:7681–7686.

16. Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. *Lancet*. 1990;335:1078–1080.
17. Cassol E, Cassetta L, Rizzi C, Alfano M, Poli G. M1 and M2a polarization of human monocyte-derived macrophages inhibits HIV-1 replication by distinct mechanisms. *J Immunol*. 2009; 182:6237–6246.
18. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A*. 2003;55:61–70.
19. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol*. 2007;8:942–949.
20. Gül A, Tugal-Tutkun I, Dinarello CA, et al. Interleukin-1β-regulating antibody XOMA 052 (gevokizumab) in the treatment of acute exacerbations of resistant uveitis of Behçet's disease: an open-label pilot study. *Ann Rheum Dis*. 2012;71: 563–566.
21. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. *Nature*. 2011;469:221–225.
22. Li N, Ragheb K, Lawler G, et al. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem*. 2003;278:8516–8525.
23. Li Y, Trush MA. Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production. *Biochem Biophys Res Commun*. 1998; 253:295–299.
24. Schroder K, Tschopp J. The inflammasomes. *Cell*. 2010;140: 821–832.
25. Kaneko F, Oyama N, Yanagihori H, Isogai E, Yokota K, Oguma K. The role of streptococcal hypersensitivity in the pathogenesis of Behçet's Disease. *Eur J Dermatol*. 2008;18:489–498.
26. Nishida T, Hirayama K, Nakamura S, Ohno S. Proliferative response of CD8+ gamma delta+ T cells to Streptococcus sanguis in patients with Behçet's disease. *Ocul Immunol Inflamm*. 1998;6:139–144.
27. Pay S, Simşek I, Erdem H, Dinç A. Immunopathogenesis of Behçet's disease with special emphasis on the possible role of antigen presenting cells. *Rheumatol Int*. 2007;27:417–424.
28. Choi B, Lee ES, Sohn S. Vitamin D3 ameliorates herpes simplex virus-induced Behcet's disease-like inflammation in a mouse model through down-regulation of Toll-like receptors. *Clin Exp Rheumatol*. 2011;29(4 suppl 67):S13–S19.
29. Wiken M, Grunewald J, Eklund A, Wahlstrom J. Higher monocyte expression of TLR2 and TLR4, and enhanced pro-inflammatory synergy of TLR2 with NOD2 stimulation in sarcoidosis. *J Clin Immunol*. 2009;29:78–89.
30. Ren W, Hu L, Hua F, Jin J, Wang Y, Zhu L. Myeloid differentiation protein 2 silencing decreases LPS-induced cytokine production and TLR4/MyD88 pathway activity in alveolar macrophages. *Immunol Lett*. 2011;141:94–101.
31. Evereklioglu C, Er H, Türköz Y, Cekmen M. Serum levels of TNF-alpha, sIL-2R, IL-6, and IL-8 are increased and associated with elevated lipid peroxidation in patients with Behçet's disease. *Mediators Inflamm*. 2002;11:87–93.
32. Pay S, Erdem H, Pekel A, et al. Synovial proinflammatory cytokines and their correlation with matrix metalloproteinase-3 expression in Behçet's disease. Does interleukin-1beta play a major role in Behçet's synovitis? *Rheumatol Int*. 2006;26:608–613.
33. Filippin LI, Vercelino R, Marroni NP, Xavier RM. Redox signalling and the inflammatory response in rheumatoid arthritis. *Clin Exp Immunol*. 2008;152:415–422.
34. Gilgun-Sherki Y, Melamed E, Offen D. The role of oxidative stress in the pathogenesis of multiple sclerosis: the need for effective antioxidant therapy. *J Neurol*. 2004;251:261–268.
35. Burek CL, Rose NR. Autoimmune thyroiditis and ROS. *Autoimmun Rev*. 2008;7:530–537.
36. Bulua AC, Simon A, Maddipati R, et al. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). *J Exp Med*. 2011;208:519–533.
37. Kastner DL, Aksentijevich I, Goldbach-Mansky R. Autoinflammatory disease reloaded: a clinical perspective. *Cell*. 2010;140: 784–790.
38. Lacroix-Lamande S, d'Andon MF, Michel E, et al. Downregulation of the Na/K-ATPase pump by leptospiral glycolipoprotein activates the NLRP3 inflammasome. *J Immunol*. 2012; 188:2805–2814.
39. Segovia J, Sabbah A, Mgbemena V, et al. TLR2/MyD88/NF-kappaB pathway, reactive oxygen species, potassium efflux activates NLRP3/ASC inflammasome during respiratory syncytial virus infection. *PLoS One*. 2012;7:e29695.