

Expression and Function of Inducible Costimulator on Peripheral Blood CD4⁺ T Cells in Behçet's Patients with Uveitis: A New Activity Marker?

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PURPOSE. Inducible costimulator (ICOS) is an important costimulatory molecule involved in T-cell activation. In this study, the role of ICOS in the pathogenesis of uveitis in Behçet's disease (BD) was investigated.

METHODS. Peripheral blood mononuclear cells (PBMCs) were obtained from BD patients with uveitis in the active or remission phase and in healthy subjects. Total RNA was isolated from PBMCs, and mRNA expression was analyzed on an oligonucleotide microarray. ICOS expression on CD4⁺ T cells was determined by flow cytometry, and the functional costimulatory effect of ICOS/B7RP-1 interaction was assessed on stimulation with concanavalin A (conA) or IRBP in the presence or absence of anti-ICOS mAb.

RESULTS. As the result of microarray analysis, ICOS in PBMCs showed the greatest difference in expression in BD patients with uveitis compared with healthy control subjects. ICOS expression on CD4⁺ T cells in BD patients with uveitis was significantly higher than that in healthy individuals, both before and after conA stimulation. Among the BD patients, ICOS expression on CD4⁺ T cells was significantly higher in those with active uveitis than in those with remitted uveitis. Blockade of ICOS/B7-related protein-1 (B7RP-1) interaction by anti-ICOS mAb significantly decreased IFN- γ , IL-17, and TNF- α production by PBMCs when stimulated with conA or IRBP in BD with active uveitis.

CONCLUSIONS. High ICOS expression in BD patients with uveitis contributed to the upregulation of IFN- γ , IL-17, and TNF- α production, suggesting that abnormal ICOS costimulation may play an immunopathologic role in the pathogenesis of uveitis in BD. (*Invest Ophthalmol Vis Sci.* 2010;51:5099-5104) DOI: 10.1167/iovs.10-5286

Endogenous uveitis such as Behçet's disease (BD), Vogt-Koyanagi-Harada syndrome, sympathetic ophthalmia, birdshot retinochoroidopathy, and sarcoidosis is a potentially

blinding disease in humans and is responsible for 10% to 15% of the acquired blindness in Japan.¹ Although endogenous uveitis covers a spectrum of clinical entities, all forms are believed to share immunohistologic similarities characterized by the infiltration of mainly T cells. Behçet's disease (BD) is a systemic inflammatory disease characterized by oral and genital ulcers as well as ocular, cutaneous, arthritic, vascular, and neurologic lesions.² Many factors have been implicated in the development of inflammation and severity of disease. Although genetic factors, infections, and autoimmune responses have been implicated in BD, much about its pathogenesis remains uncertain.³⁻⁵ An increasing number of reports indicate that aberrant cellular immunity, such as pathogenic CD4⁺ T-cell[b]-mediated autoimmunity via the Th1/Th17 pathway, plays a key role in the pathophysiological process in BD with uveitis,⁶⁻¹⁰ although the mechanisms of ocular inflammation in BD remain largely unknown.

During recent years, the understanding of immunologic mechanisms involved in uveitis has advanced greatly through investigations of experimental autoimmune uveoretinitis (EAU), an animal model of human uveitis that can be induced by immunization of susceptible animals with interphotoreceptor retinoid-binding protein (IRBP) or with an eye-specific retinal antigen or by adoptive transfer of CD4⁺ T cells specific for retinal antigens.¹¹ EAU resembles certain human uveitic conditions in various aspects^{12,13} and the major mediator is the CD4⁺ T cell.

It is well known that T-cell activation requires the integration of at least two distinct signals. Signal one results from antigen-specific T-cell receptor engagement by MHC class 2-bound peptide presented by antigen-presenting cells (APCs). Signal two arises from engagement of costimulatory molecules expressed by antigen-presenting cells and T cells.^{14,15} Although CD28 regulation has substantial effects on immunity, its function appears to reside predominantly in the control of primary, but not secondary, immune responses in various autoimmune diseases.¹⁶⁻¹⁸ The CD28 homolog inducible costimulator (ICOS) has recently been identified as a novel member of the CD28 costimulator family and is expressed by activated T cells in both humans and mice.^{19,20} The ICOS ligand, B7-related protein (B7RP-1), also known as B7 homologous protein (B7h), is constitutively expressed by dendritic cells, macrophages, and B cells, and the degree of expression is increased by IFN- γ , TNF- α , and IL-17.²⁰⁻²³ Discovery of the ICOS/B7RP-1 pathway has led to considerable interest in the physiologic and pathologic functions of this pathway. A series of reports support the concept that ICOS is critical in the regulation of Th2 responses.^{19,20,24,25} However, recent murine studies indicate that ICOS also regulates Th1- and Th17-mediated diseases, such as experimental allergic encephalomyelitis

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TABLE 1. Patient Characteristics

Disease Activity	Subjects (n)	Age (y)	Male/Female
Ocular Behçet			
Active	17	36.5 ± 10.1	13/4
Inactive	18	45.7 ± 15.0	14/4
Healthy individuals	21	42.7 ± 16.6	16/5

(EAE), collagen-induced arthritis, acute allograft rejection, and inflammatory bowel disease.²⁶⁻²⁹ Recently, activation of T cells linked to the expression of costimulatory molecules has been reported in various animal models, including EAU.^{18,30-34} These results prompted us to analyze the expression of costimulatory molecules in ocular BD.

To identify new genes that may cause or contribute to the disease process of ocular BD, we used cDNA microarrays that provide the expression profile of more than 54,000 genes, some of which are immune-related, whereas others are involved in the cell cycle, cell growth, intracellular signaling, cellular adhesion, and transport, and we compared the expression profiles of healthy individuals and patients. One of the genes found to be upregulated in patients with ocular BD is ICOS, an activation marker expressed on activated T cells that binds B7RP-1-expressing monocytes. The engagement of ICOS with B7RP-1 along with an appropriate antigen provide a positive signal that promotes T-cell differentiation, cytokine secretion, and effector function in the absence of CD28.³⁵ This process has been demonstrated in vivo in a murine EAU model, and we have shown that ICOS protein expressed on infiltrating CD4⁺ T cells and B7RP-1 expressed on infiltrating APCs are upregulated directly after disease onset.³⁰ Therefore, we speculated that in active BD, pathogenic CD4⁺ T cells that infiltrate the eye express ICOS in the inflamed eye and would be an appropriate target for the treatment of human ocular BD. Moreover, we have demonstrated that blockade of the ICOS/B7RP-1 costimulatory pathway inhibits ocular inflammation in the effector phase of murine EAU.³⁰ Therefore, to investigate the role of ICOS in the pathogenesis of ocular BD, we assessed its expression on peripheral blood CD4⁺ T cells and functional roles in patients with ocular BD. The results suggest that upregulation of ICOS is associated with the pathogenesis of uveitis in BD.

MATERIALS AND METHODS

PBMC Samples

Thirty-five patients with BD (27 men and 8 women, mean age; 37.8 ± 11.1 years) were enrolled in the study (Table 1). All patients fulfilled the diagnostic criteria for ocular BD proposed by the International Study Group for BD. Other etiologies were excluded after a complete ocular and systemic work-up. The samples from the patients were classified as active or inactive intraocular inflammation, according to the presence or absence of active ocular inflammation on the day of sample collection, as previously described.³⁶ Briefly, uveitis in the active phase (active intraocular inflammation) was defined by the presence of any of the following ocular inflammation findings observed by ophthalmoscope: three or more infiltrating cells in the anterior chamber, worsening vitreous opacity, and retinal exudates. Uveitis in the remission phase (inactive ocular inflammation) was defined as the absence of any of the signs of active disease. Seventeen patients had recurrent uveitis, and active intraocular inflammation was observed in blood samples obtained at the Ophthalmic Center of Tokyo Medical University Hospital. Eighteen patients had inactive ocular BD. Twenty-one healthy individuals (16 men and 5 women, mean age; 42.7 ± 16.6 years) served as control subjects. Patients who had used oral or intravenous corticosteroids or immunosuppressive agents such as cyclo-

sporine were excluded. All subjects gave informed consent before sample acquisition. The study was approved by the ethics committee of the Tokyo Medical University and complied with the guidelines in the Declaration of Helsinki for research involving human subjects.

Heparinized blood samples were obtained from patients and healthy individuals, and PBMCs were isolated within 2 hours by density gradient centrifugation. The PBMCs were washed twice and resuspended at 1×10^6 cells/mL in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 1 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin). Cell suspension was dispensed in 24-well plates (Falcon; BD Biosciences, Mountain View, CA) at 1 mL/well and incubated with or without concanavalin A (ConA; 10 µg/mL) for 12 hours.

Analysis of Gene Expression with cDNA Array

Gene expression profile of human PBMCs was analyzed by microarray, as described previously.³⁷ Briefly, total RNA was isolated from pooled PBMCs obtained from seven patients with BD and active uveitis by an extraction method (Isogen Nippon Gene, Tokyo, Japan), and portions (2 µg) of the preparation were subjected to amplification of mRNA with T7 RNA polymerase. Biotin-labeled cRNA (10 µg) synthesized from the amplified RNA was subjected to hybridization with the Human Whole Genome Bioarray chip (Amersham Biosciences, Piscataway, NJ) that contains oligonucleotides corresponding to a total of approximately 55,585 human genes. Detection and digitization of hybridization signals were performed (G2565; Agilent Technologies, San Diego, CA) and analyzed (CodeLink Expression Analysis software version 4.0; Amersham Biosciences). The microarray data of various costimulatory molecules are summarized in Table 2. Expression levels of each gene were automatically normalized to the median of all spot intensities.

Reagents

FITC-conjugated anti-human CD4 (L3T4) was purchased from eBioscience (San Diego, CA), and anti-human ICOS (DX29) from BD Pharmingen (San Diego, CA). ConA was obtained from Vector Laboratories (Burlingame, CA).

Preparation of IRBP

Fresh swine IRBP was purified according to the method described by Fukai et al.³⁸ with some modification. Briefly, frozen retinas were thawed in 0.03 M disodium monopotassium phosphate buffer (PB), after which the retinas were removed by centrifugation at 10,000g for 30 minutes. Saturated ammonium sulfate (pH 7.2) was added to the supernatant until 50% saturation was reached, and the mixture was left overnight at 4°C. After centrifugation, the precipitate was dissolved in PB and used as the crude antigen preparation. IRBP was isolated from the preparation according to the method of Redmond et al.³⁹ Briefly, the preparation was purified successively by conA-Sepharose affinity chromatography (HPLC) and ion-exchange HPLC. IRBP in the eluted

TABLE 2. Genes Up- or Downregulated on PBMC with or without ConA in Ocular Behçet Patients

Genes of Costimulatory Molecules	Intensity Difference		GenBank Accession Number
	(-)	ConA for 12 h	
<i>ICOS</i>	1.134	3.226	NM_012092
<i>CD40L</i>	0.274	0.031	NM_000074
<i>4-1BB</i>	-0.004	2.151	NM_001561
<i>CTLA4</i>	-0.231	0.229	NM_005214
<i>CD28</i>	-0.265	-1.125	NM_006139
<i>CD27</i>	-1.382	-1.834	NM_001242
<i>PD-1</i>	-0.110	-0.167	NM_005018

CTLA4, cytotoxic T-lymphocyte-associated antigen 4; PD-1, programmed death-1.

fractions was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and binding of [3 H]retinol. Purification of IRBP to homogeneity, as assessed by SDS-PAGE and silver staining, was accomplished by size-exclusion HPLC in the presence of glycerol.

Immunofluorescence and Flow Cytometry

The results of ICOS gene expression obtained from microarray were confirmed by flow cytometry. PMBCs were isolated by density gradient centrifugation of heparinized blood samples obtained from patients and healthy individuals. Each PMBC sample was divided into two aliquots: one for direct flow cytometry analysis of ICOS expression and the other for flow cytometry analysis after activation with conA at 10 μ g/mL for 12 hours. ICOS analysis was performed by using the following procedures: A cocktail of FITC-conjugated anti-CD4 and PE-conjugated anti-ICOS was added to the PMBCs. After the cells were washed with PBS, the stained cells (live-gated based on forward- and side-scatter profiles and propidium iodide exclusion) were passed through a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA) and the results analyzed (CellQuest program; BD Biosciences). ICOS expression on human CD4⁺ T cells was calculated by gating for the CD4⁺ T cell population, and 10,000 cells were analyzed in each experiment.

Cytokine Assay

Purified PMBCs (5×10^5 /well) from BD patients with active uveitis were cultured in 96-well microtiter plates, with or without stimulation with conA (3 μ g/mL) or IRBP (10 μ g/mL) plus anti-ICOS mAb (10 μ g/mL) or control IgG at 37°C for 48 hours. The unstimulated PMBCs served as negative control samples. After stimulation, cell-free supernatants were collected at 48 hours and assayed for IFN- γ , TNF- α , IL-2, and IL-6 by cytometric bead array (CBA) kits (BD PharMingen) and IL-17 by ELISA (Human IL-17 Quantikine ELISA kit; R&D Systems, Minneapolis, MN), according to the manufacturers' instructions. The minimum levels detected by CBA kits in either the culture supernatant or serum/plasma were 2.6 pg/mL for IL-2, 3.0 pg/mL for IL-6, 7.1 pg/mL for IFN- γ , and 2.8 pg/mL for TNF- α ; the minimum level in the IL-17 ELISA was 7.8 pg/mL.

In a preliminary study, serial concentrations of conA (1, 3, 5, and 10 μ g/mL) or IRBP (1, 5, and 10 μ g/mL) were used in the assays. The results showed that optimal concentrations of cytokines were obtained at a concentration of 3 μ g/mL for conA and 10 μ g/mL for IRBP, as previously described.⁴⁰ These concentrations were therefore used in subsequent experiments.

Statistical Analysis

Data were analyzed (JMP 5; SAS Institute, Inc., Cary, NC) and the results expressed as the mean \pm SD. Statistical analysis was performed by using a paired or unpaired *t*-test. *P* < 0.05 was considered significant.

RESULTS

Analysis of Microarray Results in BD Patients with Active Uveitis

To identify the genes involved in the pathogenesis of ocular BD, we compared phosphorescent images of the arrays hybridized with cDNA probes generated from RNA preparations from BD patients with active uveitis and healthy individuals. The array membrane that we used contained 360 positive bacterial controls and 384 negative controls. All positive controls were detected on the microarray. No signals were observed for the negative spots, indicating that the hybridization was highly specific. The DNA screening can evaluate more than 54,000 genes for a single sample. Among these genes, we focused on the immune-related ones, especially those of costimulatory molecules. Among the seven genes of costimulatory molecules, ICOS showed the greatest difference in expression in BD patients with active uveitis compared with that in healthy control

subjects (Table 2), supporting the reports by others and us that ICOS plays an important role in autoimmune diseases.^{28,41,42}

ICOS Expression on CD4⁺ T Cells of BD Patients with Uveitis

Flow cytometry was performed to confirm the results of gene array analysis. ICOS expression (median fluorescence intensity [MFI] of ICOS on CD4⁺ T cells; Fig. 1A, positive rates of ICOS⁺CD4⁺ T cells; Fig. 1B) on freshly isolated CD4⁺ T cells from BD patients with uveitis (active or inactive) was significantly upregulated compared with that in healthy individuals. On activation by conA for 12 hours, ICOS expression on CD4⁺ T cells was significantly higher in BD patients with uveitis compared with healthy individuals. In the absence of conA, ICOS expression on CD4⁺ T cells was also significantly higher in BD patients with uveitis than in healthy individuals. These results suggest that higher ICOS expression on CD4⁺ T cells in BD patients with uveitis may be involved in the pathogenesis of ocular BD.

Comparison of ICOS Expression on CD4⁺ T Cells in BD Patients with Active or Inactive Uveitis

Next, we compared ICOS expression on CD4⁺ T cells in BD patients with active or inactive uveitis. ICOS expression (MFI of ICOS on CD4⁺ T cells, Fig. 2A; positive rates of ICOS⁺CD4⁺ T cells, Fig. 2B) on freshly isolated CD4⁺ T cells from BD patients with active uveitis was significantly upregulated compared with that in those with inactive uveitis. After 12 hours of incubation, ICOS expression (MFI and percentage of CD4⁺ T cells expressing ICOS) was similar in BD patients with active or inactive uveitis in the presence or absence of conA. These results suggest that higher ICOS expression on CD4⁺ T cells in BD patients with uveitis is closely linked with ocular BD. These results suggest that higher ICOS expression on CD4⁺ T cells in BD patients with uveitis is closely linked with the active disease status.

Cytokine Production Stimulated with ConA and Uveitogenic Antigen

To determine whether ICOS has functional costimulatory activity, we first measured the amounts of pathogenic Th1 and Th17 cytokines (IFN- γ , IL-17, TNF- α , IL-6, and IL-2) produced by non-specific stimulation (conA) of PMBCs obtained from BD patients with active uveitis. When ICOS costimulation was blocked with anti-ICOS mAb, the amounts of IFN- γ , IL-17, and TNF- α (Figs. 3A-C) produced by PMBCs were both significantly reduced.

Since PMBCs from BD patients are known to be sensitized to the two most uveitogenic retina-specific antigens, S-antigen and IRBP,¹ we then examined the amounts of pathogenic Th1 and Th17 cytokines produced by stimulation with swine IRBP protein as the specific antigen. Figures 3F-I show the results obtained from five or six representative BD patients with active uveitis. When ICOS costimulation was blocked with anti-ICOS mAb, the amounts of IFN- γ and IL-17 production by IRBP stimulation were reduced in a manner similar to that caused by nonspecific stimulation, except TNF- α . Although no significant production of IL-6 and -2 was observed, both conA- and IRBP-induced productions of IL-6 were apparently reduced, at least in part, by blockade of ICOS costimulation. Thus, these results suggest that signaling through ICOS costimulation is involved in the production of pathogenic Th1 and Th17 cytokines by PMBCs when stimulated with nonspecific or retina-specific antigens.

DISCUSSION

The precise pathogenesis of uveitis associated with BD is unknown. Accumulated data obtained from animal models such as EAU suggest that Th1 and Th17 cells have major roles

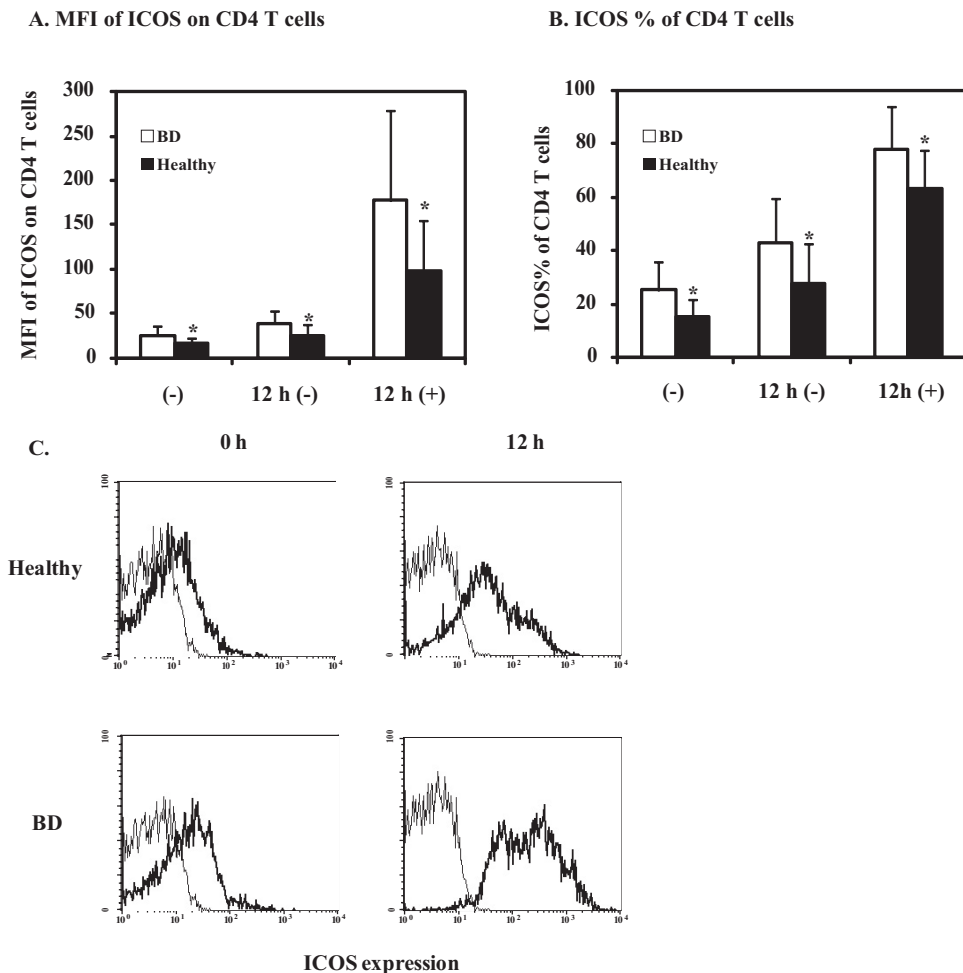


FIGURE 1. Flow cytometry of ICOS-expressing CD4⁺ T cells in PBMCs of patients with BD with uveitis and in healthy individuals. (A) MFI of ICOS on CD4⁺ T cells and (B) proportions of ICOS-expressing CD4⁺ T cells from BD patients with uveitis (*n* = 35) and normal subjects (*n* = 21). Bars show the mean ± SD (**P* < 0.05). (-): freshly isolated PBMCs; 12 h (-): incubated without conA; 12 h (+): incubated for 12 hours with conA. (C) Representative flow cytometry profiles of PBMCs from BD patients with uveitis and normal subject before and after incubation with conA for 12 hours.

in its pathogenesis.^{43,44} Studies on autoantigen-induced cellular immune responses in BD patients with uveitis mainly used assays that measure the lymphoproliferative response and cytokine productions.^{1,8,45} Previous studies showed that Th1 and Th17 cytokine production by polyclonally stimulated PBMCs and activated T cells was significantly elevated in BD patients with active uveitis compared with patients without active uveitis or controls.^{9,46} We therefore further examined the influence on IFN- γ , IL-17, TNF- α , IL-6, and IL-2 production by PBMCs after nonspecific or antigen-specific stimulation when we blocked ICOS costimulation with anti-ICOS mAb. All these findings suggest that the ICOS costimulatory pathway is asso-

ciated with active uveitis in BD patients and that this pathway has a positive costimulatory role in enhancing the production of Th1 and Th17 cytokines, which are important mediators in the pathogenesis of active uveitis in BD patients. Studies on the manipulation of this pathway may validate its role and also provide a new strategy for the treatment of this disease.

We have previously shown that blockade of the ICOS⁺ costimulatory pathway has an ameliorating effect during the effector phase of EAU, by suppressing the expansion and effector function of pathogenic Th1 cells.³⁰ Considering that blocking Abs to IL-17 ameliorates murine EAU,⁴⁷ our data suggest that anti-ICOS mAb may mitigate ocular inflammation

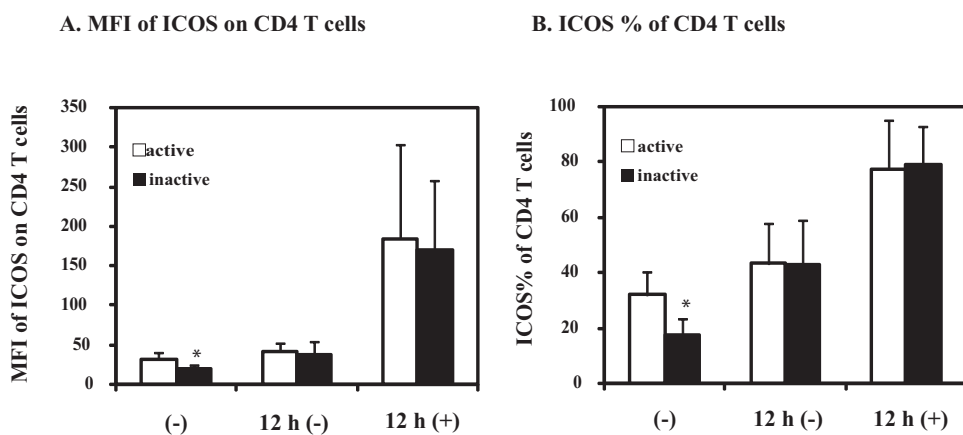


FIGURE 2. Comparison of ICOS expression on CD4⁺ T cells in BD patients with active or inactive uveitis. (A) MFI of ICOS on CD4⁺ T cells and (B) proportions of ICOS-expressing CD4⁺ T cells from BD patients with active or inactive uveitis (*n* = 17 and *n* = 18, respectively). Bars show the mean ± SD (**P* < 0.05). Labeling is as described in Figure 1.

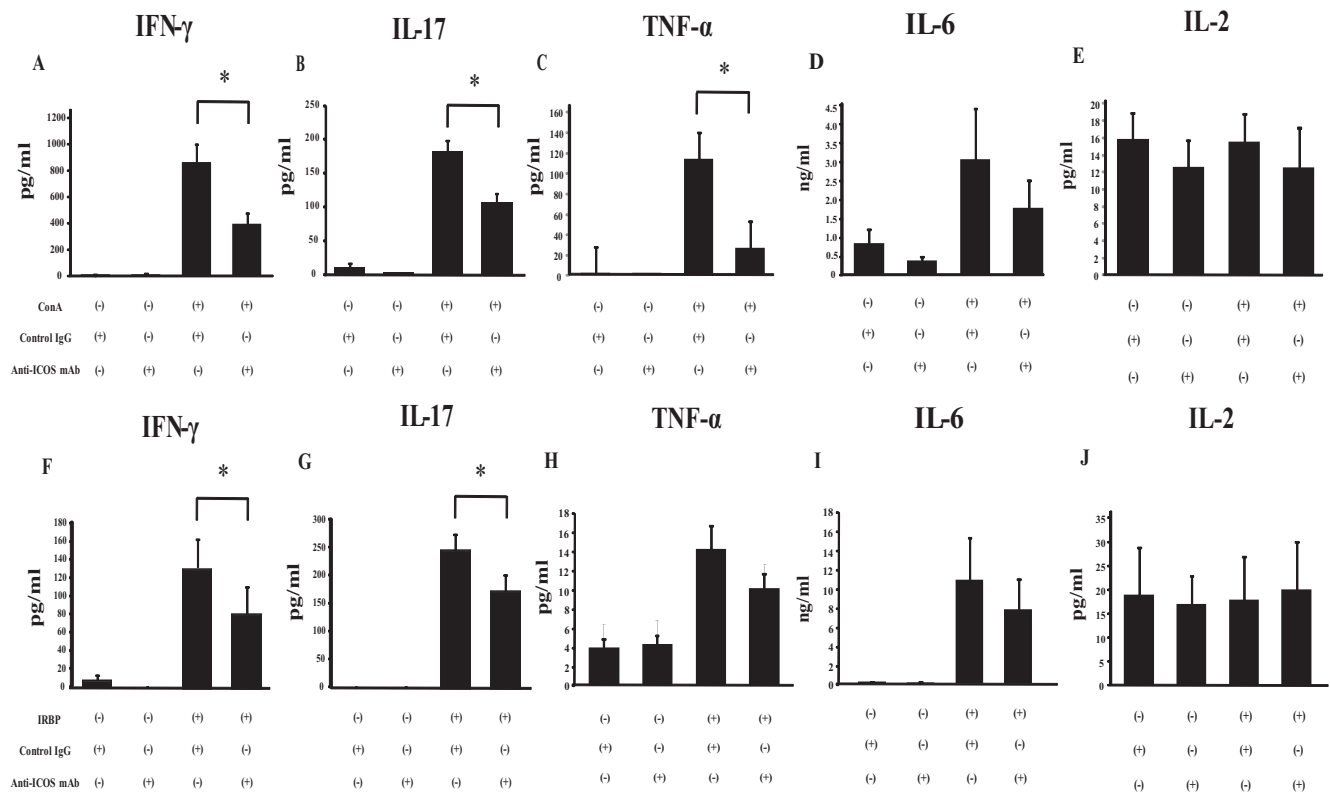


FIGURE 3. Blockade of ICOS costimulation reduced the production of Th1 and Th17 cytokines by PBMCs. PBMCs were isolated from BD patients with active uveitis and cultured in triplicate with nonspecific and antigen-specific stimulation. The levels of IFN- γ , IL-17, TNF- α , IL-6, and IL-2 were measured in the culture supernatants by cytometric bead analysis. The ICOS costimulatory pathway blocked production of IFN- γ (A), IL-17 (B), TNF- α (C), IL-6 (D), and IL-2 (E) by nonspecific stimulation. PBMCs were cultured with conA (3 μ g/mL) in the presence or absence of blocking Abs to ICOS. The equivalent isotype Abs were used for a negative control. Each value represents the mean \pm SD of five or six individuals ($^*P < 0.05$). (F–J) The ICOS costimulatory pathway blocked production of IFN- γ (F), IL-17 (G), TNF- α (H), IL-6 (I), and IL-2 (J) by antigen specific stimulation. PBMCs were cultured with IRBP (10 μ g/mL) in the presence or absence of blocking Abs to ICOS. The equivalent isotype Abs were used for a negative control. Each value represents the mean \pm SD of results for five or six individuals ($^*P < 0.05$).

and systemic autoimmune responses in BD patients with active uveitis by inhibiting Th1 and Th17 cytokine production.

In this study, ICOS was markedly upregulated in CD4⁺ T cells from BD patients with uveitis. Both the proportion of ICOS-expressing cells and percentage of ICOS expression in freshly isolated CD4⁺ T cells were significantly higher in BD patients with active uveitis than in those with inactive uveitis. ICOS expression on CD4⁺ T cells from BD patients with uveitis may play an immunopathologic role in ocular inflammation. It is important to evaluate the activity of ocular BD. However, currently there is no reliable laboratory marker. C-reactive protein and erythrocyte sedimentation rate, which indicate inflammatory processes nonspecifically, probably cannot reflect the continuously changing magnitude of inflammatory activity in BD.^{48,49} On the other hand, the level of ICOS expression on CD4⁺ T cells from BD patients with active uveitis may be a sensitive index for evaluating the disease activity of ocular BD. Our results also suggest that ICOS expression on circulating peripheral CD4⁺ T cells would be a useful marker for monitoring the therapeutic effects of conventional treatment in patients with BD with active uveitis. In the case of systemic lupus erythematosus (SLE), expression of ICOS on peripheral T cells of patients with active disease was significantly reduced after treatment with high-dose corticosteroid, a strong inhibitor of lymphocyte activation, and the treatment almost completely abolishes the induction of ICOS with ICOS costimulation.⁵⁰ Results in a recent study of T-cell-mediated autoimmune disease suggested that ICOS expression reflects the aggressiveness of autoreactive effector T cells.⁵¹ The

higher ICOS expression in BD patients with active uveitis may be associated with a unique immunopathogenesis and the more recurrent or chronic nature of BD. Further studies with prospective follow-up are needed to address this possibility.

In conclusion, our data provide additional evidence of the potential utility of ICOS expression on CD4⁺ T cells as a marker of disease activity and as a promising therapeutic target for ocular BD via its inhibition of Th1 and Th17 cytokines. As the population studied was small and heterogeneous, further studies are needed to confirm the findings.

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References

1. Yamamoto JH, Minami M, Inaba G, Masuda K, Mochizuki M. Cellular autoimmunity to retinal specific antigens in patients with Behçet's disease. *Br J Ophthalmol.* 1993;77:584–589.
2. O'Duffy JD. Behçet's disease. *Curr Opin Rheumatol.* 1994;6:39–43.
3. Direskeneli H. Behçet's disease: infectious aetiology, new autoantigens, and HLA-B51. *Ann Rheum Dis.* 2001;60:996–1002.
4. Mizuki N, Ota M, Yabuki K, et al. Localization of the pathogenic gene of Behçet's disease by microsatellite analysis of three different populations. *Invest Ophthalmol Vis Sci.* 2000;41:3702–3708.
5. Kitaichi N, Kotake S, Sasamoto Y, et al. Prominent increase of macrophage migration inhibitory factor in the sera of patients with uveitis. *Invest Ophthalmol Vis Sci.* 1999;40:247–250.

6. de Smet MD, Dayan M. Prospective determination of T-cell responses to S-antigen in Behçet's disease patients and controls. *Invest Ophthalmol Vis Sci.* 2000;41:3480-3484.
7. Frassanito MA, Dammacco R, Cafforio P, Dammacco F. Th1 polarization of the immune response in Behçet's disease: a putative pathogenetic role of interleukin-12. *Arthritis Rheum.* 1999;42:1967-1974.
8. Zhao C, Yang P, He H, et al. S-antigen specific T helper type 1 response is present in Behçet's disease. *Mol Vis.* 2008;14:1456-1464.
9. Chi W, Zhu X, Yang P, et al. Upregulated IL-23 and IL-17 in Behçet patients with active uveitis. *Invest Ophthalmol Vis Sci.* 2008;49:3058-3064.
10. Choi SC, Kim KD, Kim JT, et al. Expression of human NDRG2 by myeloid dendritic cells inhibits down-regulation of activated leukocyte cell adhesion molecule (ALCAM) and contributes to maintenance of T cell stimulatory activity. *J Leukoc Biol.* 2008;83:89-98.
11. Caspi RR, Sun B, Agarwal RK, et al. T cell mechanisms in experimental autoimmune uveoretinitis: susceptibility is a function of the cytokine response profile. *Eye.* 1997;11:209-212.
12. Hirose S, Tanaka T, Nussenblatt RB, et al. Lymphocyte responses to retinal-specific antigens in uveitis patients and healthy subjects. *Curr Eye Res.* 1988;7:393-402.
13. Nussenblatt RB, Kuwabara T, de Monasterio FM, Wacker WB. S-antigen uveitis in primates: a new model for human disease. *Arch Ophthalmol.* 1981;99:1090-1092.
14. Chambers CA. The expanding world of co-stimulation: the two-signal model revisited. *Trends Immunol.* 2001;22:217-223.
15. Coyle AJ, Gutierrez-Ramos JC. The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. *Nat Immunol.* 2001;2:203-209.
16. Salomon B, Bluestone JA. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu Rev Immunol.* 2001;19:225-252.
17. Chitnis T, Najafian N, Abdallah KA, et al. CD28-independent induction of experimental autoimmune encephalomyelitis. *J Clin Invest.* 2001;107:575-583.
18. Silver PB, Hathcock KS, Chan CC, Wiggert B, Caspi RR. Blockade of costimulation through B7/CD28 inhibits experimental autoimmune uveoretinitis, but does not induce long-term tolerance. *J Immunol.* 2000;165:5041-5047.
19. Hutloff A, Dittrich AM, Beier KC, et al. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature.* 1999;397:263-266.
20. Yoshinaga SK, Whoriskey JS, Khare SD, et al. T-cell co-stimulation through B7RP-1 and ICOS. *Nature.* 1999;402:827-832.
21. Swallow MM, Wallin JJ, Sha WC. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNF α . *Immunity.* 1999;11:423-432.
22. Aicher A, Hayden-Ledbetter M, Brady WA, et al. Characterization of human inducible costimulator ligand expression and function. *J Immunol.* 2000;164:4689-4696.
23. Bauquet AT, Jin H, Paterson AM, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol.* 2009;10:167-175.
24. Dong C, Juedes AE, Temann UA, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature.* 2001;409:97-101.
25. Tafuri A, Shahinian A, Bladt F, et al. ICOS is essential for effective T-helper-cell responses. *Nature.* 2001;409:105-109.
26. Ozkaynak E, Gao W, Shemmeri N, et al. Importance of ICOS-B7RP-1 costimulation in acute and chronic allograft rejection. *Nat Immunol.* 2001;2:591-596.
27. Rottman JB, Smith T, Tonra JR, et al. The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. *Nat Immunol.* 2001;2:605-611.
28. Iwai H, Kozono Y, Hirose S, et al. Amelioration of collagen-induced arthritis by blockade of inducible costimulator-B7 homologous protein costimulation. *J Immunol.* 2002;169:4332-4339.
29. Schaefer JS, Montufar-Solis D, Vigneswaran N, Klein JR. ICOS promotes IL-17 synthesis in clonally intraepithelial lymphocytes in IL-10 $^{-/-}$ mice. *J Leukoc Biol.* 2010;87(2):301-308.
30. Usui Y, Akiba H, Takeuchi M, et al. The role of the ICOS/B7RP-1 T cell costimulatory pathway in murine experimental autoimmune uveoretinitis. *Eur J Immunol.* 2006;36:3071-3081.
31. Xing L, Yang P, Wu C, et al. Inducible co-stimulator (ICOS) is upregulated in experimental autoimmune uveoretinitis. *Graefes Arch Clin Exp Ophthalmol.* 2006;244(12):1650-1658.
32. Shao H, Fu Y, Liao T, et al. Anti-CD137 mAb treatment inhibits experimental autoimmune uveitis by limiting expansion and increasing apoptotic death of uveitogenic T cells. *Invest Ophthalmol Vis Sci.* 2005;46:596-603.
33. Bagenstose LM, Agarwal RK, Silver PB, et al. Disruption of CD40/CD40-ligand interactions in a retinal autoimmunity model results in protection without tolerance. *J Immunol.* 2005;175:124-130.
34. Namba K, Ogasawara K, Kitaichi N, et al. Amelioration of experimental autoimmune uveoretinitis by pretreatment with a pathogenic peptide in liposome and anti-CD40 ligand monoclonal antibody. *J Immunol.* 2000;165:2962-2969.
35. Carreno BM, Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu Rev Immunol.* 2002;20:29-53.
36. Fujimori K, Ohi K, Takeuchi M, et al. Circulating neutrophils in Behçet disease is resistant for apoptotic cell death in the remission phase of uveitis. *Graefes Arch Clin Exp Ophthalmol.* 2008;246:285-290.
37. Abcouwer SF, Marjon PL, Loper RK, Vander Jagt DL. Response of VEGF expression to amino acid deprivation and inducers of endoplasmic reticulum stress. *Invest Ophthalmol Vis Sci.* 2002;43:2791-2798.
38. Fukui T, Okada AA, Sakai J, et al. The role of costimulatory molecules B7-1 and B7-2 in mice with experimental autoimmune uveoretinitis. *Graefes Arch Clin Exp Ophthalmol.* 1999;237:928-933.
39. Redmond TM, Wiggert B, Robey FA, et al. Isolation and characterization of monkey interphotoreceptor retinoid-binding protein, a unique extracellular matrix component of the retina. *Biochemistry.* 1985;24:787-793.
40. Takeuchi M, Usui Y, Okunuki Y, et al. Immune responses to interphotoreceptor retinoid-binding protein and S-antigen in Behçet's patients with uveitis. *Invest Ophthalmol Vis Sci.* 2010;51(6):3067-75.
41. Iwai H, Abe M, Hirose S, et al. Involvement of inducible costimulator-B7 homologous protein costimulatory pathway in murine lupus nephritis. *J Immunol.* 2003;171:2848-2854.
42. Totsuka T, Kanai T, Iiyama R, et al. Ameliorating effect of anti-inducible costimulator monoclonal antibody in a murine model of chronic colitis. *Gastroenterology.* 2003;124:410-421.
43. Luger D, Silver PB, Tang J, et al. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J Exp Med.* 2008;205:799-810.
44. Luger D, Caspi RR. New perspectives on effector mechanisms in uveitis. *Semin Immunopathol.* 2008;30:135-143.
45. de Smet MD, Yamamoto JH, Mochizuki M, et al. Cellular immune responses of patients with uveitis to retinal antigens and their fragments. *Am J Ophthalmol.* 1990;110:135-142.
46. Raziuddin S, al-Dalaa A, Bahabri S, Siraj AK, al-Sedairy S. Divergent cytokine production profile in Behçet's disease: altered Th1/Th2 cell cytokine pattern. *J Rheumatol.* 1998;25:329-333.
47. Zhang R, Qian J, Guo J, Yuan YF, Xue K. Suppression of experimental autoimmune uveoretinitis by anti-IL-17 antibody. *Curr Eye Res.* 2009;34:297-303.
48. Katsantonis J, Adler Y, Orfanos CE, Zouboulis CC. Adamantiades-Behçet's disease: serum IL-8 is a more reliable marker for disease activity than C-reactive protein and erythrocyte sedimentation rate. *Dermatology.* 2000;201:37-39.
49. Itoh R, Takenaka T, Okitsu-Negishi S, Matsushima K, Mizoguchi M. Interleukin 8 in Behçet's disease. *J Dermatol.* 1994;21:397-404.
50. Kawamoto M, Harigai M, Hara M, et al. Expression and function of inducible co-stimulator in patients with systemic lupus erythematosus: possible involvement in excessive interferon-gamma and anti-double-stranded DNA antibody production. *Arthritis Res Ther.* 2006;8:R62.
51. Yang JH, Zhang J, Cai Q, et al. Expression and function of inducible costimulator on peripheral blood T cells in patients with systemic lupus erythematosus. *Rheumatology (Oxford).* 2005;44:1245-1254.