Decreased ocular inflammatory attacks and background retinal and disc vascular leakage in patients with Behçet's disease on infliximab therapy

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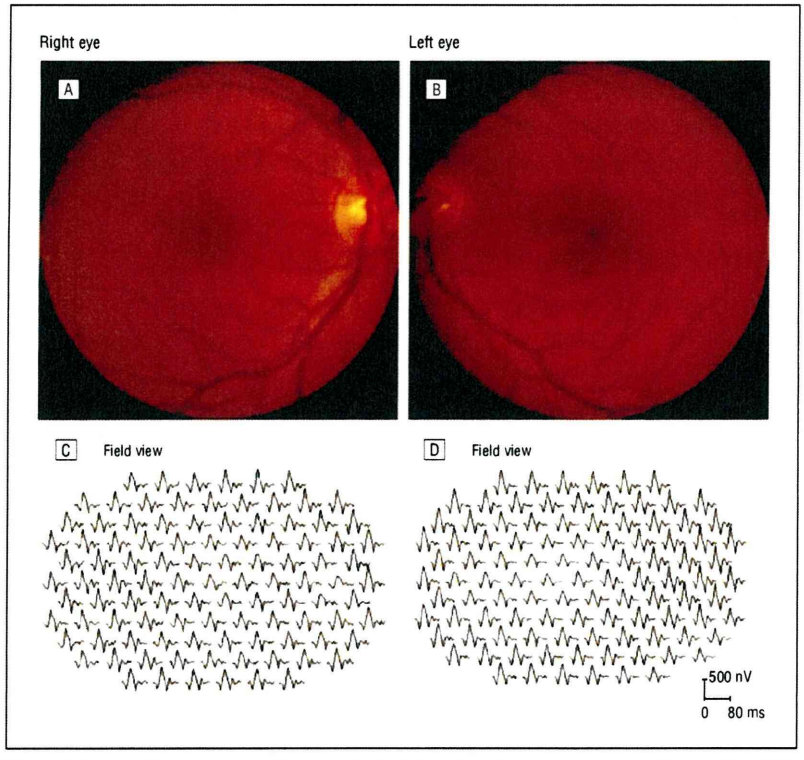

Figure 4. Fundus photographs of patient 2 at the final 4-month follow-up were unremarkable in the right eye (A) but showed reddish, oval lesions in the left eye (B). Multifocal electroretinogram demonstrated subnormal amplitudes with normal implicit times in the right eye (C), but these changes were more prominent in the region between the disc and the fovea in the left eye (D).

gional decrease in intrinsic tissue autofluorescence suggested pathologic changes at the level of the retinal pigment epithelium and possibly involving the outer retina. The additional use of mfERG highlighted the functional deficits of these patients despite better than 20/25 visual acuity. Specifically, bilateral reduction of central responses of outer retinal origin with normal implicit times was identified in both patients. This was especially helpful in identifying abnormal photoreceptor function in the asymptomatic eye of patient 2.

Our findings are consistent with prior reports of outer retinal architectural changes observed with SD-OCT in AMNR.[2,5] The addition of mfERG to precisely identify cone photoreceptor dysfunction provided a correlation of a functional deficit to the structural changes observed in the outer retina. Although we did not identify a choroidal or retinal vascular perfusion defect by fluorescein angiography or indocyanine green angiography testing, focal hypofluorescence in the region of the macular lesions was observed on fluorescein angiography of patient 2. This could represent inner choroidal ischemia or blockage of choroidal fluorescence from a focal inflammatory accumulation with resultant overlying outer retinal architectural disruption.

The term *AMNR* was originally applied to this condition because of the acute onset of presentation and the theory that the superficial macular retina was involved; given our observations correlating functional and structural aspects of this disease, the term *acute macular outer retinopathy* may be more appropriate.

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Interferon-γ Release Assay in Tuberculous Scleritis

Scleritis is a painful, often chronic, and potentially destructive ocular inflammation caused by either infectious agents or noninfectious immune reactions. Tuberculosis (TB) is one possible infectious cause of scleritis. In this report, we describe 3 patients in whom use of an interferon (IFN)-γ release assay assisted in the diagnosis of tuberculous scleritis.

Report of Cases. *Case 1.* A 29-year-old woman was referred for bilateral anterior scleritis refractory to topical corticosteroids. On examination, corrected visual acuities were 1.2 with normal intraocular pressure in both eyes. The sclera was markedly hyperemic in all 4 quadrants bilaterally (**Figure 1** A). Mild inflammatory cells were present in the anterior chambers in both eyes but the fundi were unremarkable. Laboratory investigations revealed

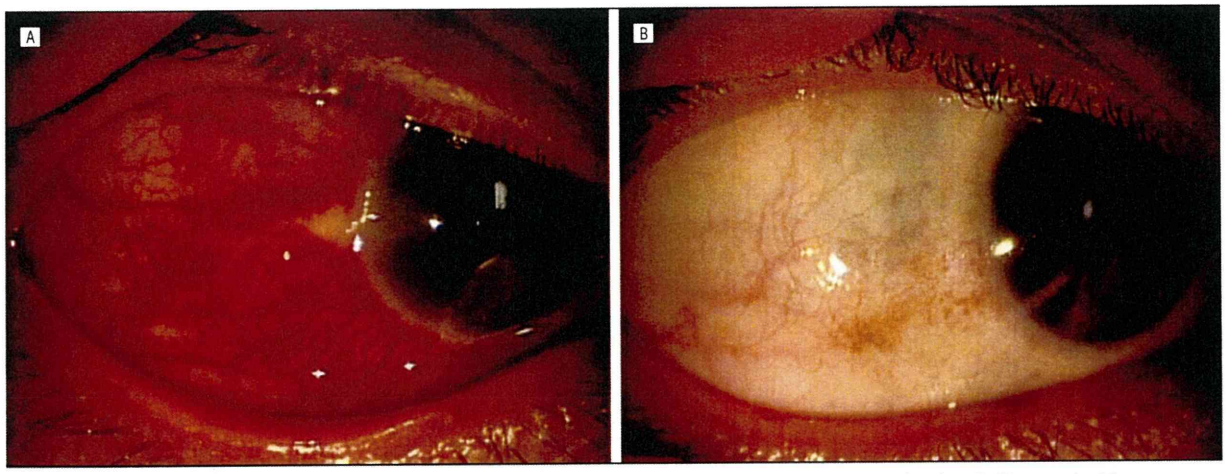

Figure 1. Slitlamp photographs for case 1. A, Slitlamp photograph of the right eye in case 1, revealing severely hyperemic sclera. B, Photograph of the same eye 1 month after initiation of antituberculosis therapy, showing a marked decrease in inflammation but some mild thinning of the sclera.

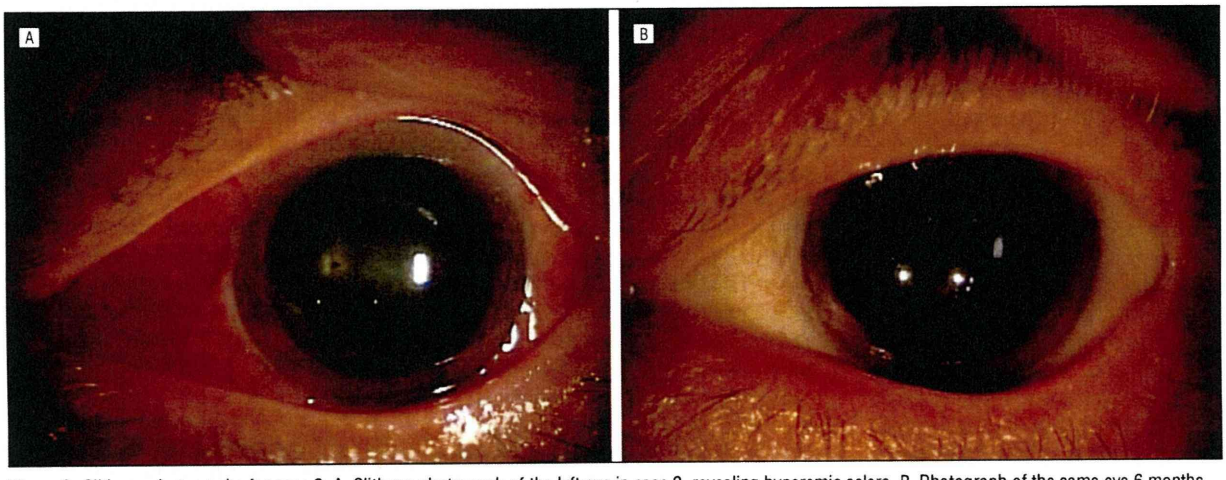

Figure 2. Slitlamp photographs for case 2. A, Slitlamp photograph of the left eye in case 2, revealing hyperemic sclera. B, Photograph of the same eye 6 months after initiation of antituberculosis therapy, showing no active inflammation.

normal chemistry results and blood cell counts; however, the erythrocyte sedimentation rate was elevated at 46 mm/h and results of a tuberculin skin test (TST) were positive with 38 mm of erythema and 18 mm of induration. The initial chest radiograph was unremarkable; however, subsequent chest computed tomography revealed multiple nodular lesions in the lung parenchyma. Results of testing using QuantiFERON-TB 2G (QFT) (Cellestis, Carnegie, Australia), an IFN-γ release assay, were found to be positive (defined as IFN-γ levels >0.35 IU/mL by the laboratory). The patient was diagnosed with tuberculous scleritis and treated with a 3-drug regimen (isoniazid, 300 mg/d; rifampicin, 450 mg/d; and ethambutol hydrochloride, 750 mg/d) for the initial 2 months, followed by the same doses of isoniazid and rifampicin for an additional 7 months. The bilateral scleritis resolved within 1 month after initiating anti-TB treatment (Figure 1B). No recurrences of scleritis were observed over 6 months of follow-up since completing therapy.

Case 2. A 72-year-old man was referred for unilateral scleritis refractory to topical corticosteroid therapy. His history was remarkable for having been diagnosed with unilateral uveitis in the left eye in 2005 at another hospital, the details of which were unknown. On examination, the corrected visual acuities were 0.5 OU, with normal intraocular pressure. The right eye was entirely normal, but the left eye exhibited marked scleral hyperemia in all 4 quadrants (**Figure 2**A). In addition, although the vitreous was clear, retinal and choroidal folds in the superior macular area were noted. Laboratory investigation results were positive for the presence of perinuclear antineutrophil cytoplasmic antibodies and an elevated erythrocyte sedimentation rate of 41 mm/h. The TST results showed 40 mm of erythema and 10 mm of induration; however, chest radiography examination was normal. Results of subsequent QFT testing were positive. An orbital computed tomographic scan confirmed diffuse thickening of the posterior eye wall and unilateral anterior and posterior scleritis was diagnosed, possibly due to TB. The patient was treated with a 4-drug regimen (isoniazid, 300 mg/d; rifampicin, 450 mg/d; ethambutol hydrochloride, 750 mg/d; and pyrazinamide, 1500 mg/d) for the first 2 months, fol-

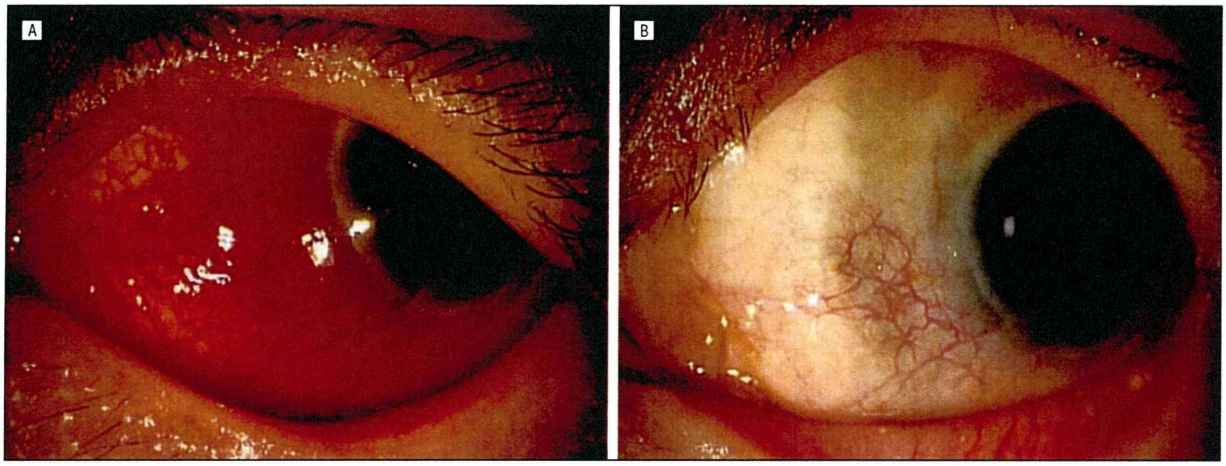

Figure 3. Slitlamp photographs for case 3. A, Slitlamp photograph of the left eye in case 3, revealing diffuse hyperemic sclera. B, Photograph of the same eye 6 months after initiation of antituberculosis therapy, showing a marked decrease in inflammation but thinning of the sclera.

lowed by the same doses of isoniazid and rifampicin for 7 months. The scleritis improved within 2 months of initiating anti-TB therapy, and no recurrences were observed over 3 months of follow-up since completing treatment (Figure 2B).

Case 3. A 68-year-old man was referred for unilateral anterior scleritis refractory to topical corticosteroid therapy. On examination, the corrected visual acuities were 1.2, with normal intraocular pressure in both eyes. The right eye was normal, but the left eye showed marked hyperemia of the sclera in 3 quadrants (**Figure 3**A) but no anterior chamber or posterior segment inflammation. Results of blood tests were unremarkable with a normal erythrocyte sedimentation rate. The chest radiograph was normal; however, the TST results showed 40 mm of erythema and 8 mm of induration. This raised our suspicion of possible TB, and QFT testing was subsequently performed and results were found to be positive. The patient received the same 4-drug regimen as in case 2, and the inflammation responded favorably within 1 month of starting anti-TB treatment, with no recurrences noted (Figure 3B).

Comment. Pulmonary TB remains a major health concern in Japan, with an estimated incidence of 21 in 100 000 and an estimated prevalence of 35 767 for 2007, roughly 4 to 5 times that for the United States.[1] Extrapulmonary disease accounts for about 20% of TB cases and can involve virtually any organ of the body, including the lymph nodes, central nervous system, skeletal system, pleura, liver, kidney, and skin.[2] Although the TST has long been used to support a diagnosis of active or latent TB, it is believed to have low specificity in populations that routinely receive BCG vaccination such as in Japan.[3] Recently, IFN-γ release assays have been developed to aid in the diagnosis of TB. The QFT assay measures IFN-γ released when whole blood is stimulated with 2 synthetic peptides, the early secreted antigenic target 6-kDa protein (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10), both found in *Mycobacterium tuberculosis* but not in the BCG vaccine or in the vast majority of atypical mycobacterium.[4]

In the present report, the TST results were positive or with induration large enough to raise our suspicion of TB and warrant additional QFT testing. We have previously reported that the rate of TST result positivity (induration >10 mm) was roughly 20% among patients with uveitis referred to our Ocular Inflammation Service.[3] Although the TST result positivity rate is not known for our patient population with scleritis, our experience in uveitis strongly suggests that a positive TST result alone is insufficient to diagnosis tuberculous ocular inflammation. Recently, Ang and colleagues[5] have demonstrated that the combination of a positive TST result and a positive QFT result increases the accuracy of diagnosing tuberculous uveitis. The present report also supports the use of both TST and QFT in the diagnosis of tuberculous scleritis.

In summary, our 3 patients presented with scleritis refractory to topical corticosteroid therapy, standard community-based treatment for this condition in Japan. Based on positive TST and QFT results, anti-TB therapy was initiated, with all patients exhibiting improvement of inflammation within 1 to 2 months. Our experience highlights the difficulty in assessing TB as a possible cause of ocular inflammation and the potential of IFN-γ release assays for assisting in that assessment.

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Ophthalmological Numismatics

Born in Zagreb, Kurt Hühn (1875-1963) trained in Vienna and then returned to Zagreb in 1900 where he practiced ophthalmology at the Sisters of Mercy Hospital, becoming the Director of the hospital in 1935, a post he held until his retirement in 1945. One of the first to use x-ray equipment in Croatia, he also published extensively in Croatian medical journals.

In 1925, a lifetime portrait medal by the Croatian artist Ivo Kerdić of 61 mm diameter was struck in bronze to honor Hühn for 25 years of medical service. The obverse depicts Hühn's bust facing right. The reverse depicts Hühn on the left operating on the eye of a young female patient who is seated on the right.

Courtesy of: Jay M. Galst, MD, Clinical Associate Professor, New York Medical College, and Peter van Alfen, PhD, Associate Curator, American Numismatic Society.
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Intravitreal Bevacizumab for Iris Metastasis of Small-Cell Lung Carcinoma with Neovascular Glaucoma

Metastasis to the iris is rare, with the majority of cases originating in carcinomas of either the breast or lungs.[1] Small-cell lung carcinoma (SCLC) is characterized by rapid growth and early metastasis.[2] Bevacizumab is a monoclonal antibody against vascular endothelial growth factor (VEGF), and is approved in Japan for the treatment of metastatic carcinoma of the colon and rectum, and for non-SCLC. We report a case in which presumed iris metastasis complicated by neovascular glaucoma in a patient with SCLC responded to intravitreal injection of bevacizumab.

Case Report

A 52-year-old man presented to the Kyorin Eye Center complaining of blurred vision OD for 1 month. The patient had been diagnosed 14 months earlier with SCLC (lung biopsy specimen in Fig. 1), and a short time before admittance had undergone chemotherapy (five cycles of cisplatin/etoposide and two cycles of amrubicin) for multiple metastases in the adrenal gland and abdominal lymph nodes, as well as radiotherapy for brain metastasis. He was, however, currently not receiving any specific cancer treatment. On examination his visual acuity was 1.2 OU, and intraocular pressure (IOP) was 19 mmHg OD and 15 mmHg OS. Slit-lamp biomicroscopy showed 1+ anterior chamber cells OD and an inferonasal iris mass, associated with peripheral anterior synechia and neovascularization of the iris (Fig. 2A). The fundus OD and the left eye were normal.

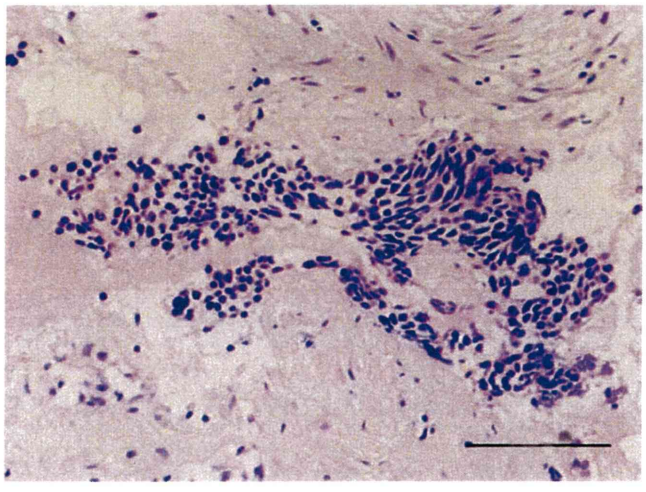

Figure 1. Histopathology from a lung biopsy specimen showing malignant cells consistent with small-cell lung carcinoma (bar = 100 μm).

The iris mass was presumed to be metastasis from SCLC, and was noted to increase in size with a rise in IOP to 36 mmHg over a 6-week period. Despite therapy with topical 0.5% timolol maleate, 1.0% brinzolamide, and 0.005% latanoprost, and oral acetazolamide 500 mg/day, the IOP continued to be poorly controlled and the patient complained of ocular pain. After informed consent, a single injection of 1.25 mg of bevacizumab (Avastin, Genentech, San Francisco, CA, USA) was injected intravitreally OD for the purpose of treating the neovascular glaucoma. The use of intravitreal bevacizumab received prior approval from the Kyorin University Hospital Ethics Committee. Two weeks after the bevacizumab injection, the iris tumor had decreased in size, the iris neovascularization had resolved, and IOP was 18 mmHg (Fig. 2B). At that time, the patient underwent additional chemotherapy with irinotecan hydrochloride hydrate for the presumed iris metastasis as well as for treatment of other metastases. Three months after the bevacizumab injection, examination showed inferonasal iris atrophy with no recurrence of neovascularization (Fig. 2C). However, continued use of antiglaucoma medication was required to keep the IOP within normal range, presumably owing to the development of peripheral anterior synechia over one-half of the angle. Eight months after the bevacizumab injection, the patient died from complications of obstructive jaundice resulting from SCLC metastasis to the lymph nodes in the hepatic hilum.

Comment

Treatment of iris metastasis generally consists of chemotherapy, sometimes in combination with radiation therapy.[1] In this report, successful short-term regression of a presumed iris metastasis and improved control of secondary neovascular glaucoma in a patient with metastatic SCLC was achieved by intravitreal injection of bevacizumab, prior to additional chemotherapy. As far as we are aware, this represents the first report of the use of intravitreal bevacizumab for SCLC metastasis to the eye. Recently, a phase II trial examining the combination of systemic bevacizumab and chemotherapy in patients with SCLC showed improved overall survival compared to controls who received chemotherapy alone.[3] Furthermore, Kuo and colleagues reported the successful use of intravitreal bevacizumab for choroidal metastasis from colorectal carcinoma,[4] and Bianciotto and colleagues reported that intravitreal bevacizumab in combination with plaque radiotherapy resulted in rapid resolution of neovascular glaucoma associated with iris melanoma.[5] These reports, together with our experience, highlight the potential for intravitreal bevacizumab in

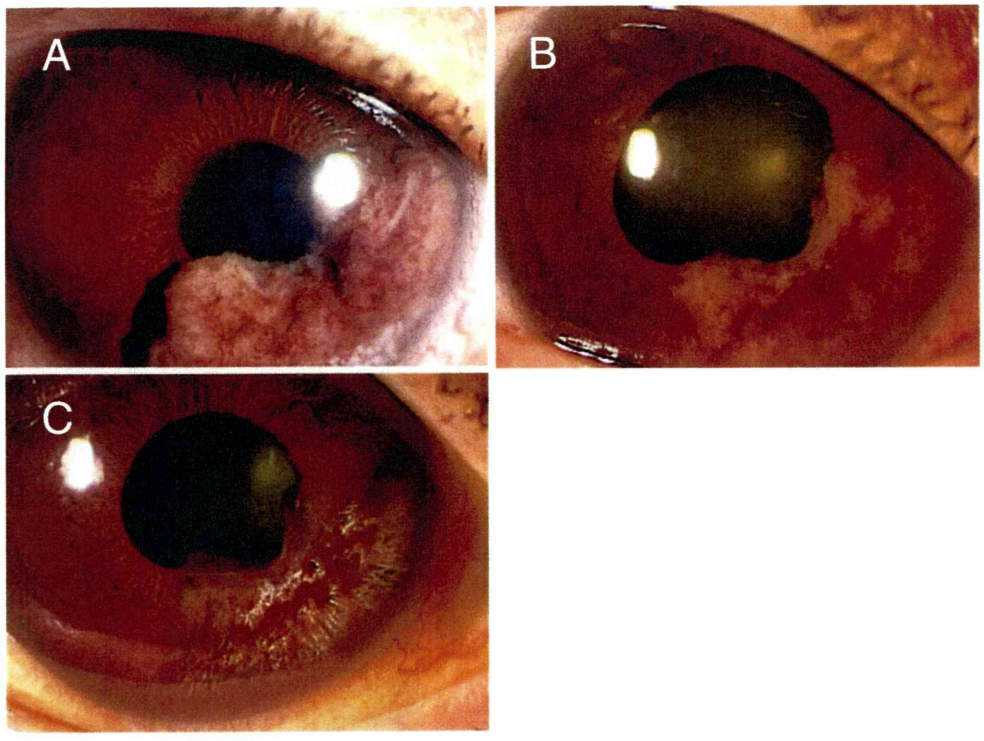

Figure 2. **A** A tumor was observed in the inferonasal iris, with peripheral anterior synechia and neovascularization of the iris and angle present. **B** Two weeks after a single intravitreal injection of 1.25 mg bevacizumab, the tumor was noted to have regressed considerably in size, with concomitant resolution of the iris neovascularization. **C** Three months after the intravitreal bevacizumab and after additional chemotherapy, iris atrophy was noted where the original tumor had been.

treating various intraocular tumors, specifically intraocular metastasis from SCLC, in the setting of secondary neovascular glaucoma.

Keywords: bevacizumab, iris tumor, neovascular glaucoma, small-cell carcinoma, VEGF

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Spectral-Domain Optical Coherence Tomography for Three-Dimensional Visualization of the Detached Membrane in Valsalva Retinopathy

Valsalva retinopathy occurs in patients with a history of increased intrathoracic pressure, and is characterized by hemorrhagic detachment of the internal limiting membrane (ILM), preretinal hemorrhage, or vitreous hemorrhage.[1]

Several reports have described the time-domain optical coherence tomography (TD-OCT) features of premacular hemorrhage and the preretinal membrane in patients with Valsalva retinopathy.[2,3] However, the information is of limited use because of the relatively slow scan speed and low axial resolution of TD-OCT. Spectral-domain optical coherence tomography (SD-OCT) achieves higher imaging speeds as well as a higher signal-to-noise ratio than TD-OCT, and can produce three-dimensional (3D) data.

To our knowledge, this is the first report on the 3D visualization of the plane structure of a detached membrane examined by SD-OCT.

als between arms. There were no statistically significant ($p < 0.05$) differences between candesartan and placebo in any comparison. In DIRECT-Protect 2, restricting analysis to subjects over age 50 did not alter results. There were too few older subjects in the other two studies to analyse. Also, separating the cohorts between smokers and nonsmokers produced similar results.

Candesartan does not have any effect on dry eye in any of the three DIRECT studies in contrast to a halving of risk with ACE inhibition in the BDES.(Moss et al. 2004) While candesartan has effects similar to ACE inhibitors on control of hypertension and inflammation, the effect of ACE inhibitors on dry eye may be separate from these processes.

Differences between the studies are that DIRECT included only people with diabetes (Chaturvedi et al. 2008; Sjolie et al. 2008) who were younger than BDES participants, whose mean age was 65 years (Moss et al. 2000), accounting for a lower frequency of dry eye than the 14.4% in the BDES. However, we know of no evidence suggesting the effects of the medications would be different for these reasons. Also, sample sizes of the DIRECT studies are adequate to detect an effect similar to Beaver Dam.

A limitation of DIRECT is that dry eye was not determined prior to treatment. However, owing to the randomization, it is unlikely there were large differences between treatment arms. Certainly, other baseline characteristics were evenly balanced.

In conclusion, there is no evidence that candesartan prevents occurrence of dry eye.

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Spontaneous closure of a recurrent myopic macular hole previously repaired by pars plana vitrectomy

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Editor,

Macular hole (MH) formation is a well-known complication of high myopia. Pars plana vitrectomy (PPV) is the preferred treatment, although myopic holes have poorer anatomical and visual prognoses compared to non-myopic eyes (Kumagai et al. 2001). Spontaneous closure is an infrequent phenomenon, and even more uncommon in recurrent holes that have been previously repaired by vitrectomy. Here, we describe a case of spontaneous closure of a recurrent myopic MH that had been closed after previous PPV.

A 55-year-old myopic Japanese man was referred for the treatment of MH in the right eye. His ocular history was significant for MH in the left eye that had undergone PPV 4 years previously with unsuccessful closure. On examination, the best-corrected

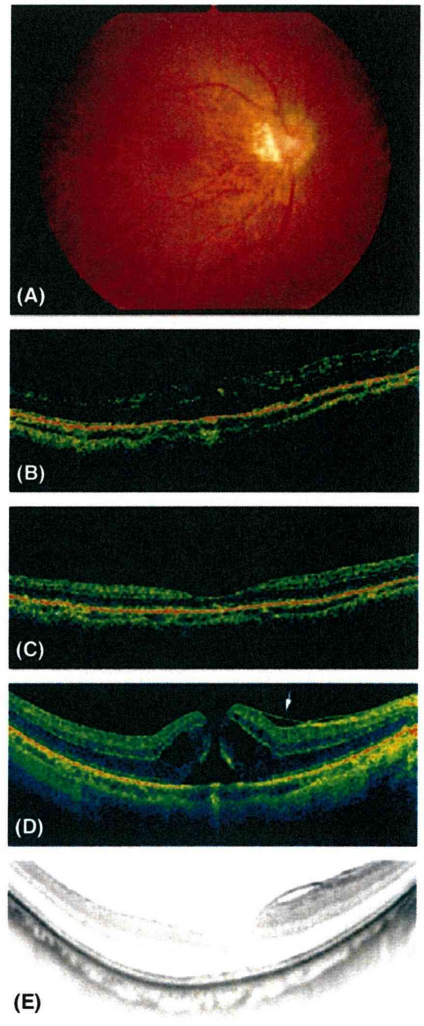

Fig. 1. Serial horizontal optical coherence tomography (OCT) scans illustrating the spontaneous closure of a recurrent myopic macular hole (MH) in a previously vitrectomized eye. (A) Preoperative colour fundus photograph of the right eye demonstrating a MH and myopic changes that include tilted disc, peripapillary atrophy, and shallow posterior staphyloma. Peripheral lattice degeneration was also noted. (B) OCT showing the initial full thickness MH with perifoveal cystic spaces [Stratus OCT™; Carl Zeiss Meditec, Inc., Dublin, CA, USA]. (C) OCT taken 4 weeks after pars plana vitrectomy showed normalization of the foveal contour [Stratus OCT™; Carl Zeiss Meditec, Inc.]. (D) Recurrence of the MH was noted 4 years after initial closure. The perifoveal internal limiting membrane was partially elevated (arrow) [Cirrus™ HD-OCT 4000; Carl Zeiss Meditec, Inc., Dublin, CA, USA]. (E) OCT performed 6 weeks later showed that the MH had spontaneously closed [Spectralis® OCT; Heidelberg Engineering, Heidelberg, Germany].

visual acuities (VAs) were 0.4 OD and 0.1 OS. The spherical equivalent refractive errors were −7.25 OD and −8.75 OS. Examination of the right eye revealed 2+ nuclear sclerosis cataract and a small MH. Myopia-related changes including a tilted disc, peripapillary atrophy, a shallow posterior staphyloma, and lattice degeneration were also observed (Fig. 1A). Optical coherence tomography (OCT) confirmed the presence of a stage 3 MH with perifoveal cystic changes (Fig. 1B), but no evidence of epiretinal membrane (ERM).

Phacoemulsification, intraocular lens implantation, and PPV with 20% SF_6 gas tamponade were performed. At the time of posterior vitreous detachment (PVD) induction, small peripheral retinal breaks occurred which were treated with endolaser. Internal limiting membrane (ILM) peeling was not performed as complete PVD had been successfully induced. Postoperatively, the MH was noted to be closed and the VA improved to 1.0 (Fig. 1C).

Four years later, the patient presented complaining of 6 months of new blurry vision in the right eye. Examination revealed a VA of 0.4 OD and recurrent stage 4 MH with perifoveal cystic changes and partial elevation of the ILM, without evidence of ERM or bridging glial tissue (Fig. 1D). Repeat PPV was scheduled; however, at a preoperative visit 6 weeks later the hole was noted to be spontaneously closed (Fig. 1E) with VA improvement to 1.2. The MH has remained closed for seven additional months, and the VA has remained stable at 1.2.

Spontaneous closure of recurrent MH after vitrectomy is a rare phenomenon. To our knowledge, only five cases have been reported: three with OCT substantiation (Shaikh & Garretson 2003; Gross 2005) and two without (Duker et al. 1994; Paques et al. 2000). Three cases were associated with ERM that appeared to reseal the hole presumably by contractile forces (Duker et al. 1994; Paques et al. 2000; Gross 2005). Three cases opened and closed concurrently with the remodelling of idiopathic or cataract surgery-associated cystoid macular oedema (CME) (Shaikh & Garretson 2003; Gross 2005).

The MH in our patient was associated with CME, perifoveal ILM separation, and myopia. A possible unifying mechanism for the hole formation may involve ILM contraction and consequent structural changes in the fovea associated with pre-ILM cellular proliferation not detected by OCT. Furthermore, other myopic abnormalities such as staphyloma, and vitreous-retinal forces may also be contributing to the loss of foveal integrity. In our case, iatrogenic breaks that occurred during PPV may have also induced microscopic extrafoveal membrane formation, imparting tangential tractional forces on the macula. However, we presume that any tractional forces would have been minimal, because the MH was small and subsequently closed without further surgical intervention. ILM remodelling and the increased elasticity and cellular alterations occurring after vitrectomy may have facilitated the process of spontaneous MH closure.

In conclusion, this case demonstrates that spontaneous closure of recurrent myopic MH in a previously vitrectomized eye can occur.

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Early onset giant retinal tear after posterior chamber phakic IOL

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Editor,

Myopic eyes have a higher incidence of retinal complications compared with emmetropic eyes. Posterior chamber phakic (PCP) intraocular lens (IOL) surgery offers several advantages for correction of high-degree myopia, such as reversibility and a greater amount of correction compared with corneal refractive techniques. (Zaldivar et al. 1998). There are reports of retinal detachments after phakic IOL. (Ruiz-Moreno et al. 2003 and Martínez-Castillo et al. 2005). We report a case of an early onset giant retinal tear with retinal detachment after PCP IOL implantation.

A 22-year-old man with high myopia of −16 diopters (D) in both eyes underwent PCP IOL in both eyes elsewhere. He then presented to us with an acute visual loss in his right eye, 2 weeks after the surgical procedure. Ophthalmological examination revealed best corrected visual acuity (BCVA) of CF at 1 m OD and 20/32 OS. Anterior segment examination showed a PCP IOL correctly implanted (Fig. 1A). The fundus examination of the right eye showed a giant retinal tear with retinal detachment. (Fig. 1B). Corrective vitreoretinal surgery with 5000cs silicone oil injection as endotamponade was performed. A macular scar was noted at the conclusion of surgery which affected the final visual outcome (Fig. 1C). In the follow-up examinations the retina remained totally reattached with a BCVA of 20/200 at 3 months (Fig. 1D).

Blockade of Interleukin-6 Signaling Suppresses Not Only Th17 but Also Interphotoreceptor Retinoid Binding Protein–Specific Th1 by Promoting Regulatory T Cells in Experimental Autoimmune Uveoretinitis

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Purpose. Both Th17 and Th1 cells contribute to experimental autoimmune uveoretinitis (EAU). Interleukin-6 (IL-6) blockade inhibits Th17 differentiation in EAU and potently suppresses ocular inflammation, although its effect on Th1 cells is unknown. To clarify the mechanism of IL-6 blockade, the authors investigated T helper cells with particular focus on Th1 and regulatory T cells (Treg) in EAU of IL-6 gene knockout (KO) mice.

Methods. EAU was induced in wild-type (WT) mice and in mice lacking IL-6 (IL-6KO), IL-17 (IL-17KO), and IFN-γ (GKO) on a C57BL/6 background. Clinical scores of EAU, cytokine levels in supernatants from ocular tissue homogenates, and T helper cell differentiation in lymph nodes in each mouse were examined. To study the roles of Treg cells, EAU was induced in IL-6KO mice treated with anti-CD25 monoclonal antibody (mAb) to deplete Treg cells in vivo.

Results. Inflammation was comparable between WT, IL-17KO, and GKO mice but was absent in IL-6KO mice. Th17 and interphotoreceptor retinoid binding protein (IRBP)–specific Th1 cells were increased in GKO and IL-17KO mice, respectively, whereas both populations were reduced in IL-6KO mice. Th1-dominant EAU in IL-17KO mice was suppressed by anti–IL-6R mAb treatment. Treg cell depletion in vivo induced EAU in IL-6KO mice.

Conclusions. After the induction of EAU, IL-6 deficiency resulted in the inhibition of the IRBP-specific Th1 response and enhanced the generation of IRBP-specific Treg cells. Furthermore, Treg was needed to inhibit Th1 responses and ocular inflammation in IL-6KO mice. Protective effects of IL-6 signaling blockade in EAU involve not only Th17 cell inhibition but also IRBP-specific Treg cell promotion. (*Invest Ophthalmol Vis Sci.* 2011;52:3264–3271) DOI:10.1167/iovs.10-6272

Experimental autoimmune uveitis (EAU) is a rodent model of human uveoretinitis, and recent studies have revealed that highly proinflammatory interleukin (IL)-17–producing $CD4^+$ T helper cells (Th17) play a pivotal role in the development of EAU, human uveitis,[1,2] and other experimental autoimmune diseases.[3–5]

Th17 cells are a newly identified subset of helper T cells distinct from Th1 or Th2 cells, and both IL-6 and TGF-β are required for their differentiation. On the other hand, TGF-β alone promotes naive T cells to differentiate into regulatory T cells (Treg), which are considered immunosuppressive helper T cells.[6–8] Thus Th17 and Treg cells are distinct subsets of helper T cells, and IL-6 signaling promotes Th17 cells and inhibits Treg cell differentiation.[9] From these studies, IL-6 signaling may represent a target for anti-inflammatory therapy. In fact, several studies have already reported that the anti–IL-6R monoclonal antibody (mAb) suppresses autoimmune disease models, including arthritis,[10] encephalomyelitis,[11] and uveitis,[12] by inhibiting Th17 cell development.

In our own previous studies, we studied the effect of anti–IL-6R mAb in EAU and found that antigen-specific IFN-γ–producing $CD4^+$ T helper cells (Th1) were also suppressed.[13] Before the discovery of the Th17 cell subset, Th1 cells had been considered to play a key role in EAU induction.[14,15] However, the loss of IFN-γ did not prevent EAU induction,[16,17] and the loss of IL-17 did not prevent EAU completely.[18] To explain these observations, studies have demonstrated that both Th1 and Th17 cells are redundant for the induction of EAU and that they substitute for each other.[18,19] Taken together, these published studies indicate that both Th1 and Th17 cells should be suppressed to completely suppress the inflammation of EAU and that the anti-inflammatory mechanism of IL-6 signaling blockade cannot be fully explained by mere Th17 cell inhibition. Th1 cells are understood to require IL-12 and IFN-γ for their differentiation from naive T cells and are not understood to require IL-6; rather, IL-6 is considered to suppress Th1 cell differentiation.[20,21] Therefore, the role of the IL-6 signaling blockade in the inhibition of Th1 responses in vivo remains to be explained clearly.

In the present study, we focused on Treg cells, which are promoted by the IL-6 signaling blockade, and analyzed the roles of the IL-6 signaling blockade. We found that the IL-6 signaling blockade not only inhibited Th17 cell differentiation

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but also promoted antigen-specific Treg cells, which, in turn, suppressed the inflammatory effects of antigen-specific Th1 cells. Thus, the inhibitory effect of the IL-6 blockade in the development of EAU is associated with suppression of the induction of both Th1 and Th17 cells and their dominant proinflammatory effects in this disease.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories (Yokohama, Japan). C57BL/6-background IL-6$^{-/-}$ (IL-6KO), IL-17A$^{-/-}$ (IL-17KO) mice were kindly provided by Yoichiro Iwakura (Laboratory of Molecular Pathogenesis, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan), and IFN-$\gamma^{-/-}$ (GKO) mice were described previously.[22] All mice were maintained in specific pathogen-free conditions at The National Institute of Biomedical Innovation, Osaka, Japan. Female mice (6–8 weeks of age) were used in all experiments. All animals were treated humanely, and all experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

EAU Induction

All mice were immunized with 100 μg human interphotoreceptor retinoid binding protein (IRBP) epitope contained in residue 1–20 sequences (GPTHLFQPSLVLDMAKVLLD) in 0.2 mL emulsion of complete Freund's adjuvant (CFA) supplemented with *Mycobacterium tuberculosis* H37Ra to 2.5 mg/mL. In addition, 500 ng pertussis toxin was inoculated intraperitoneally.

Clinical and Histopathologic Evaluation

Ophthalmic examinations were carried out after immunizations. Tropicamide (0.5%) was applied to the eyes to induce mydriasis, and the fundus of the eye was examined using a slit lamp microscope. Every 48 hours from 11 days to 29 days after immunization, animals were clinically assessed in a masked fashion by experienced ophthalmologists who examined them for the presence of dilatation, white focal lesions, and white linear lesions affecting blood vessels, retinal hemorrhaging, and retinal detachment. Clinical scores between 0 and 4 were assigned according to severity, as described previously,[23] with some modifications. Eyes were enucleated from each mouse at peak of EAU (17–21 days after immunization) and were embedded in optical cutting temperature compound (Tissue-Tek; Sakura Finetechnical Co. Ltd., Tokyo, Japan) or paraffin. Sections approximately 8- to 10-μm thick were stained by standard hematoxylin and eosin.

Preparation of Supernatants from Ocular Tissue Homogenates and Cytokine ELISAs

Eyes were enucleated after kill, and conjunctival tissue was removed. Remaining eye tissues including cornea, vitreous body, retina, choroids, and sclera were homogenized using a sample preparation device (BioMasher; Nippi Inc., Tokyo, Japan). Supernatants were collected after centrifugation at 14,300*g* for 1 minute, and levels of IFN-γ and IL-17 were measured by ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Intracellular Cytokine Staining Assays

Draining cervical lymph node cells were collected from three to six mice on indicated days after immunization with IRBP. For intracellular cytokine detection, cells were stimulated for 4 hours with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 750 ng/mL ionomycin in the presence of 10 μg/mL Brefeldin A (BD Biosciences, San Jose, CA). Cell surface antigens were stained with anti-CD4 antibody (Biolegend, San Diego, CA). For the detection of FoxP3$^+$ Treg, cells were stained using a FoxP3 staining kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. Antibodies anti-IFN-γ (Biolegend) and IL-17 (BD Bioscience) were used simultaneously for detecting Th1 and Th17 cells, respectively. Stained cells were analyzed by flow cytometry (FACScanto; BD Biosciences). Each fluorescence-activated cell sorting (FACS) profile was obtained using cells from one mouse.

Cell Cultures

Cells were cultured in RPMI 1640 (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Hyclone, Irvine, CA), 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan), penicillin G, and streptomycin for 72 hours.

5,6-Carboxyfluorescein Succinimidyl Ester and Enzyme-Linked Immunosorbent Assays

Draining cervical lymph node cells were collected 10 days after immunization. Cells were labeled in 3 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) and were cultured with IRBP (100 μg/mL) for 3 days, harvested, and stained with antibodies against CD4, IFN-γ, IL-17, and Foxp3 for the detection of Th1 and Th17 Treg cells or with antibodies against GITR, Foxp3, and CTLA-4 (Biolegend) for the detection of Foxp3$^-$ Treg cells and were analyzed using flow cytometry (FACSCanto; BD Biosciences). For these FACS analyses we used a Foxp3 staining kit with which we could detect both intracellular antigens, including cytokines, Foxp3, and CTLA-4, and extracellular antigens, including CD4 and CTLA-4 (which is expressed intracellularly and is also cell-surface expressed[24]). In addition, IFN-γ and IL-17 in the cell culture supernatant were measured by ELISA kits (R&D Systems).

Treatment with Anti–IL-6R Antibody

Neutralizing anti-IL-6R mAb (clone MR16-1), which is a rat IgG1 antibody against murine IL-6 receptor, was provided by Chugai Pharmaceutical Co. (Gotemba, Japan) and Oriental Yeast Co. Ltd. (Tokyo, Japan). Anti-IL-6R mAb (8 mg) or purified rat nonimmune isotype control IgG (MP Biomedicals, Solon, OH) was injected intraperitoneally on day 1 after the induction of EAU in IL-17KO and GKO mice.

Regulatory T Cell Depletion In Vivo

IL-6KO mice were administered a total of three intraperitoneal injections (100 μg/injection) of either anti-CD25 mAb (clone PC61; BD PharMingen, San Jose, CA) or purified rat nonimmune isotype control IgG (MP Biomedicals) at 48-hour intervals, as described previously.[25] EAU was induced 7 days after the completion of treatment.

Results

IL-6 Gene Knockout Inhibits the Development of EAU

A previous study by Yoshimura et al.[12] has shown that IL-6KO mice displayed weak inflammation after EAU induction whereas WT mice displayed severe inflammation. In agreement with this observation, we obtained comparable data in the present study. Histologic examination showed that a number of inflammatory cells infiltrated the eyes of WT mice and that the retinal structure was destroyed; IL-6KO mice showed almost no inflammation (Fig. 1A). In addition, IFN-γ and IL-17 cytokines in supernatants from ocular tissue homogenates were detected after the induction of EAU in WT mice but not in IL-6KO mice (Fig. 1B).

IL-6 Gene Knockout Inhibits the Development of Th17 and IRBP-Specific Th1 Cells In Vivo

Because IL-6 is essential in Th17 cell differentiation in vitro, we evaluated in vivo helper T cell differentiation in WT and IL-6KO mice by FACS. On days 0, 10, and 20 after immunization, cervical lymph node cells were collected. Th17 cells in WT

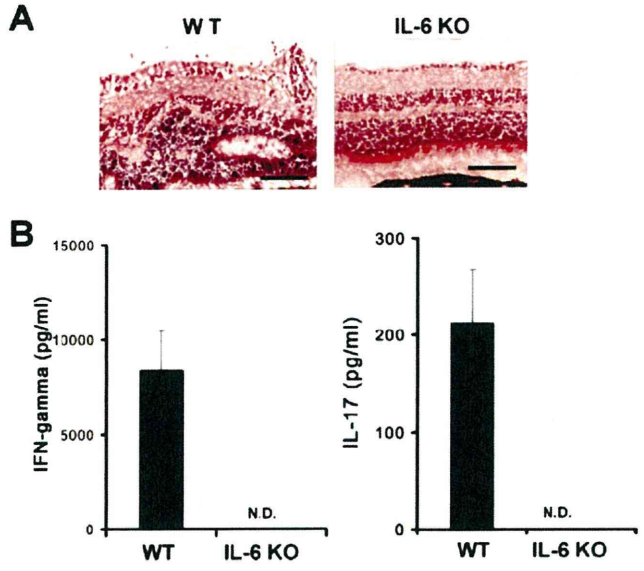

FIGURE 1. Inhibition of EAU development in IL-6KO mice. (**A**) In WT mice, a number of inflammatory cells infiltrated the eye, and the retinal structure was destroyed. In contrast, IL-6KO mice showed no inflammatory change. Scale bar, 100 μm. (**B**) Detection of IFN-γ and IL-17 levels in supernatants from ocular tissue homogenates at peak of EAU in WT mice but not in IL-6KO mice. Data show the average ± SD of three mice. N.D., not detected.

mice increased markedly on day 10 and decreased on day 20. Compared with WT mice, the development of Th17 cells in IL-6KO mice was significantly ($P < 0.05$) impaired throughout the course of EAU (Figs. 2A, 2B). In contrast, Th1 cells in both WT and IL-6KO mice increased on day 10 (Figs. 2A, 2B). As we expected, the development of Th17 cells was also impaired, but the development of Th1 cells was not impaired in vivo with the abrogation of IL-6 signaling. However, these results were obtained from whole $CD4^+$ cells in vivo. To analyze IRBP-responsive T cells, we cultured lymph node cells labeled with CFSE dye. IRBP-specific CD4 T cells were investigated by FACS by means of gating on $CFSE^{low}$ cells (Fig. 2C). In addition, cell supernatants cocultured with IRBP (10 and 100 μg/mL) were investigated by ELISA. The proportion of IRBP-specific Th17 cells decreased in IL-6KO mice, and IL-17 protein levels in cell culture supernatants significantly decreased in IL-6KO mice compared with WT mice. Interestingly, IFN-γ and IRBP-specific Th1 cells in IL-6KO mice also decreased compared with WT mice (Figs. 2C–E). From these data, we conclude that endogenous IL-6 is required for the development of both Th17 and IRBP-specific Th1 cells in vivo in EAU.

IL-17KO and GKO Mice Displayed Inflammation Similar to That of WT Mice

Because both Th1 and Th17 cells are considered to play important roles in the development of EAU,[18,19] we investigated whether mice lacking IFN-γ or IL-17 develop EAU, and we investigated T cell differentiation in EAU. Consequently, in agreement with previous reports, GKO mice showed severe inflammation comparable to that in WT mice (Fig. 3A).[16,17] IL-17KO mice displayed slightly weaker inflammation compared to that in WT mice; however, this difference did not attain statistical significance. Histologic examination revealed the infiltration of inflammatory cells and the destruction of retinal structure in IL-17KO and GKO mice, as was observed in WT mice (Fig. 3B).

IRBP-Specific Th1 or Th17 Cells Can Induce EAU

We used FACS to investigate T cell differentiation in IL-17KO and GKO mice compared with WT mice to examine Th1 and Th17 cell differentiation. IRBP-specific Th17 cells on day 10 after immunization markedly increased in GKO mice compared with WT mice (Figs. 3C, 3D). In contrast, IRBP-specific Th1 cells moderately increased in IL-17KO mice compared with WT mice by FACS analysis (Figs. 3C, 3D). Taken together, we consider that Th1 cells are dominant in IL-17KO mice and Th17 cells in GKO mice, as has been previously reported.[18]

Anti–IL-6R Antibody Suppressed Inflammation of EAU in GKO and IL-17KO Mice

Treatment with anti-IL-6R mAb in GKO and IL-17KO mice was performed to examine the role of IL-6 signaling in the development of EAU in these two mouse strains. Inflammation could be suppressed by anti-IL-6R mAb treatment in GKO mice, as was expected (Fig. 3E). Interestingly, treatment with anti-IL-6R mAb also suppressed EAU in IL-17KO mice (Fig. 3F). Given the dominant inflammatory effects of Th1 cells in the development of EAU in IL-17KO mice, anti-IL-6R mAb suppresses Th1-induced inflammatory responses in the EAU model. Based on our data, we considered that IL-6 signaling blockade inhibits both Th17 and IRBP-specific Th1 cell effects in EAU model. Thus far, there have been few studies on the relationship of IL-6 and Th1 cells in vitro and in vivo; rather, IL-6 is considered to inhibit Th1 cell differentiation.[20,21] Therefore, we next investigated the mechanisms of IRBP-specific Th1 cell inhibition in IL-6 signaling blockade.

Treg Cell Increases

Treg cells increased in the late phase of EAU in both WT and IL-6KO mice. IRBP-specific Treg cells increased in IL-6KO mice in the effector phase of EAU.

TGF-β without IL-6 promotes Treg cell differentiation, and Treg cells are reported to be immunosuppressive T cells.[7] Therefore, we hypothesized that Treg cells may play an important role in ameliorating inflammation by IL-6 signaling blockade and thus examined whether Treg cells increase with IL-6 signaling blockade. However, Treg cells in vivo on day 0 and on day 10 after immunization showed equivalent levels in both WT and IL-6KO mice (Fig. 4A) and increased on day 20 after immunization. We further investigated IRBP-specific Treg cells by coculture with IRBP. IRBP-specific Treg cells in IL-6KO mice were significantly increased compared with WT mice on day 10 (Figs. 4B, 4C). In GKO and IL-17KO mice, the IRBP-specific Treg cell population showed no significant differences compared with WT mice (Fig. 3C). To detect Treg markers other than Foxp3, we also used antibodies against GITR and CTLA-4. The expression of these two Treg markers was increased in $Foxp3^+$ cells of both WT and IL-6KO mice, and the frequency of cells expressing these markers was higher in IL-6KO mice than in WT mice (Fig. 4D).

Enhanced Inflammation in Treg Cell-Depleted IL-6KO Mice Compared with IL-6KO Control Mice

We considered that early priming of IRBP-specific Treg cells in IL-6KO mice plays a key role in inhibiting inflammation. Therefore, we hypothesized that the depletion of Treg cells in IL-6KO mice might predispose to inflammation after the induction of EAU. Treg cell-depleted mice were generated by preinjection (intraperitoneal) of anti-IL-2R (anti-CD25) mAb 1 week before EAU induction, as described previously.[25] As a negative control, purified rat IgG was used. In agreement with our hypothesis, after the induction of EAU, inflammation was induced in Treg cell-depleted mice compared with control

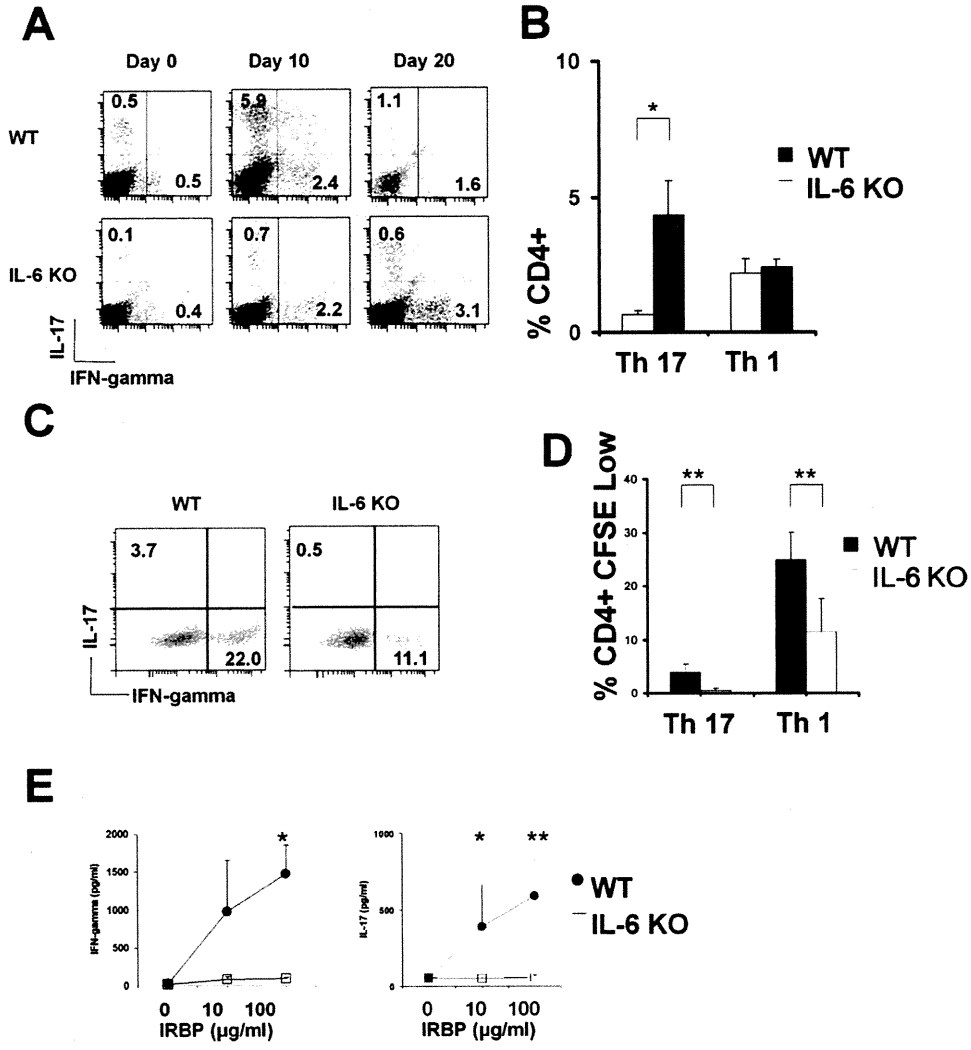

FIGURE 2. Th17 but not Th1 cells were impaired in IL-6KO mice in vivo. Differentiations in both IRBP-specific Th17 and Th1 cells were impaired in IL-6KO mice. (**A**) Intracellular cytokine staining of cells from cervical lymph nodes. Th17 cells significantly increased on day10 in WT mice; however, Th17 cells in IL-6KO mice did not proliferate on day 10 or 20. Th1 cells increased on days 10 and 20 in WT and IL-6KO mice. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A and were stained with antibodies against CD4, IFN-γ, and IL-17. All plots are gated on $CD4^+$ T cells. Values in boldface are representative data obtained from one mouse per group; all groups include at least three mice. (**B**) Frequency of $CD4^+$ $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cells in WT and IL-6KO mice on day 10. Data show the average ± SD of four to six mice. Welch's *t*-test for Th17 cells. $^*P < 0.05$. (**C**) CFSE-labeled cultured lymph node cells with 100 μg/mL IRBP were stained with CD4, IFN-γ, and IL-17. $CD4^+$ cells were gated, and $CFSE^{low}$ populations were considered as IRBP-responder cells. Values in boldface are representative data obtained from one mouse per group; both groups include six mice. (**D**) Frequency of IRBP-specific Th1 and Th17 cells in WT and IL-6KO mice. Both Th1 and Th17 cells significantly decreased in IL-6KO mice. Data showed the average ± SD of six mice. Welch's *t*-test for Th17 cells and Student's *t*-test for Th1 cells. $^{**}P < 0.01$. (**E**) Concentrations of IFN-γ and IL-17 in cell culture supernatants. Lymph nodes from WT and IL-6KO mice on day 10 were cultured with IRBP at the indicated concentrations for 3 days, and supernatants were analyzed by ELISA. Data were representative of three to six mice. Data show the average ± SD. Welch's *t*-test; $^*P < 0.05$; $^{**}P < 0.01$.

mice, as determined by analysis of EAU clinical scores (Fig. 5A). We confirmed Treg cells were significantly ($P < 0.01$) impaired in vivo in this model compared with control mice, as determined by FACS analysis of Foxp3 expression (Figs. 5B, 5C).

Increased IRBP-Specific Th1 and Th17 Differentiation in Treg Cell-Depleted IL-6KO Mice

To examine the population of Th1 and Th17 cells in Treg cell-depleted mice, lymph node cells on day 10 after immunization were analyzed. Th1 and Th17 cells in vivo were not significantly changed between Treg cell-depleted and control mice (Fig. 5B), but both IRBP-specific Th1 ($P < 0.01$) and Th17 cells ($P < 0.01$) increased significantly in Treg cell-depleted mice compared with control mice (Figs. 5D, 5E). We also confirmed that IRBP-specific Treg cells were significantly ($P < 0.01$) decreased in Treg cell-depleted mice compared with control mice (Figs. 5D, 5E). Taken together, these results suggest that Treg cell depletion by anti-CD25 mAb restored

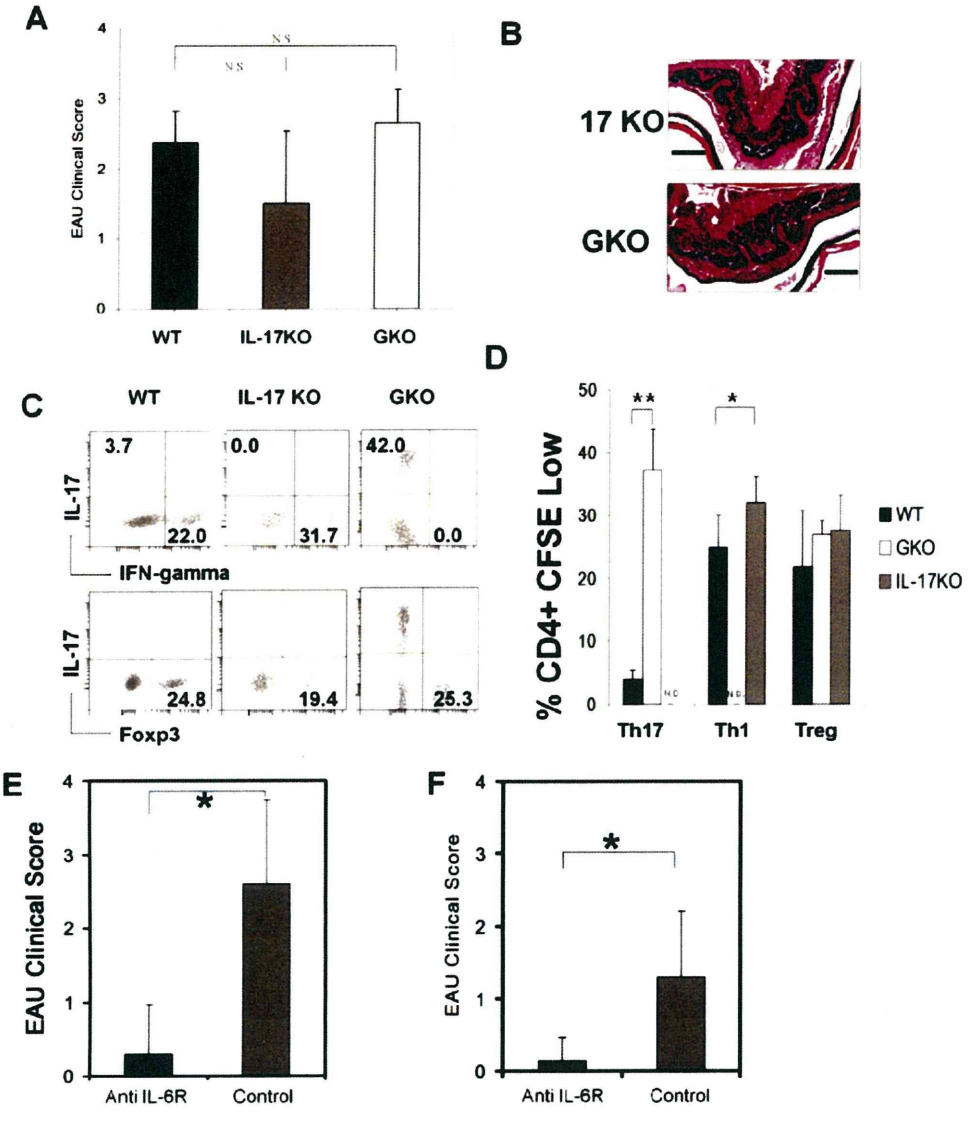

FIGURE 3. Induction of EAU in IL-17KO and GKO mice and inhibition of EAU development by IL-6 blockade therapy. **(A)** Data show the average ± SD of clinical scores of WT, IL-17KO, and GKO mice. Each group included seven to eight mice. No significant changes were seen between these three groups. N.S., not significant. **(B)** In IL-17KO and GKO mice, a number of inflammatory cells infiltrated the eye, and the retinal structure was destroyed. Scale bar, 100 μm. **(C)** Intracellular cytokine staining of three groups. Lymph node cells were CFSE labeled and cocultured with IRBP, as described. Values in boldface are representative data obtained from one mouse per group; all groups include at least three mice. **(D)** The frequency of IRBP-specific Th17, Th1, and Foxp3$^+$ Treg cells. Th17 cells in GKO mice increased greatly, and Th1 cells in IL-17KO mice increased moderately. Treg cells showed no significant change. N.D., not detected. Data show the average ± SD of at least four mice. Welch's *t*-test for Th17 cells and Treg cells and Student's *t*-test for Th1 cells. $^*P < 0.05$; $^{**}P < 0.01$. **(E, F)** Anti-IL6R mAb injection (8 mg) on day 1 after EAU induction ameliorated EAU in both GKO **(E)** and IL-17KO **(F)** mice. Mann-Whitney *U* test; $^*P < 0.05$.

inflammatory CD4 T cell profiles and ocular inflammation in IL-6KO mice. Therefore, the promotion of IRBP-specific Treg cell generation is critical for inhibiting EAU in IL-6KO mice.

DISCUSSION

In the present study, we demonstrated that genetic ablation of IL-6 signaling completely inhibited the development of EAU. In IL-6KO mice, Th17 cell differentiation and IRBP-specific Th1 response were impaired. Ablation of IL-6 signaling in EAU resulted in the promotion of IRBP-specific Treg cells, and the deletion of these Treg cells led to the restored IRBP-specific Th1 response. Our findings suggest that IRBP-specific Treg cell promotion is one of the key mechanisms of the IL-6 signaling blockade for the inhibition of inflammation in EAU.

Previous studies have demonstrated that IL-6 can inhibit Th1 cell differentiation in vitro.[20,21] However, in our previous study, we demonstrated that treatment with anti-IL-6R mAb in the EAU mouse model inhibits the IRBP-specific Th1 response in vivo.[13] In agreement with this observation, in the present study we analyzed IL-6KO mice and revealed that IRBP-specific Th1 cells are significantly decreased in IL-6KO mice compared with WT mice. Our present study confirmed that inhibition of the Th1 response by anti-IL-6R mAb treatment is not caused by the potential nonspecific effects of this antibody but by its effect on IL-6 signaling itself. In addition, we found that the administration of anti-IL-6R mAb could suppress Th1-dominant EAU in IL-17KO mice. Collectively, IL-6 is likely to be implicated in proper Th1 responses in vivo, especially in IRBP-specific Th1 responses in autoimmune disease; IL-6 may work differently on Th1 cells in vivo and in vitro.

Treg cells are immunosuppressive helper T cells[26,27] and are effective at ameliorating EAU.[28,29] As reported previously, our data showed that Treg cells are increased in the late phase of EAU (day 20) in vivo.[30] There were no differences in the frequency of total Treg cells in IL-6KO, GKO, and IL-17KO mice compared with WT mice. In contrast, IRBP-specific Treg cells in IL-6KO mice were significantly increased on day 10, implying that the increase in IRBP-specific Treg cells at an early stage is one of the key immunosuppressive mechanisms of IL-6 signaling blockade.

To further examine whether Treg cells in IL-6KO mice are critical for the inhibition of EAU, we tried to deplete their Treg cells in vivo. IL-2 is essential in the maintenance of Treg function,[31–33] and anti-IL-2R (CD25) mAb injection is effective in in vivo Treg cell depletion.[25] As expected, anti-CD25 mAb treatment resulted in the reduction of Treg cells and the exacerbation of EAU in IL-6KO mice, indicating that Treg cells in

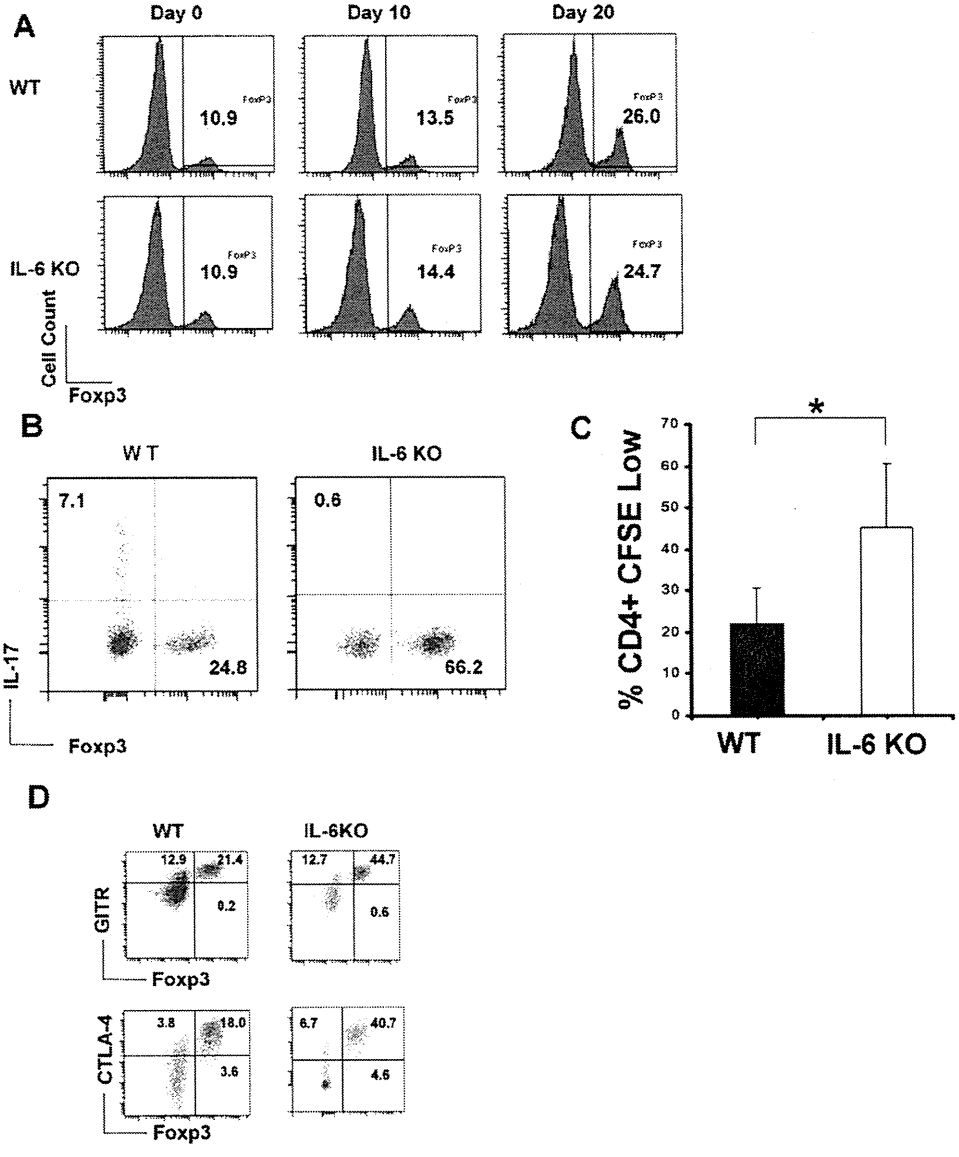

FIGURE 4. Treg cells in vivo in IL-6KO mice were comparable with those in WT mice. IRBP-specific Treg cells increased in IL-6KO mice on day 10. (**A**) Intracellular cytokine staining for CD4 and Foxp3 revealed that Treg cells increased in the late phase of EAU in both WT and IL-6KO mice. No significant difference in Treg cell populations was observed between these two mouse strains. (**B**) Lymph node cells on day 10 were labeled by CFSE and cocultured with IRBP, as described. $CD4^+$ cells were gated. Values in boldface are representative data obtained from one mouse per group; all groups include six mice. (**C**) Frequency of IRBP-specific Treg cells in IL-6KO mice was significantly higher than that of WT mice. Data show the average ± SD of six mice. Student's *t*-test; $^*P < 0.05$. (**D**) Lymph node cells on day 10 were labeled by CFSE and cocultured with IRBP and $CFSE^{low}$; $CD4^+$ cells were gated. Values in boldface are representative data obtained from one mouse per group; all groups include at least three mice.

these mice play an important role in the inhibition of EAU. However, although anti-CD25 mAb treatment is widely used for Treg cell depletion,[9,25] most—but, importantly, not all—$CD4^+Foxp3^+$ cells are also $CD25^+$.[31] Thus it should be noted that there exists some degree of limitation in the total depletion of Treg cells by this standard methodology.

Interestingly, the frequency of Th1 and Th17 cells in draining lymph nodes was not significantly different between Treg cell-depleted and -nondepleted IL-6KO mice; however, IRBP-specific Th1 and Th17 cells were increased in Treg cell-depleted mice. In accordance with our finding, Treg cells are considered to suppress Th1 cells directly.[6,30,34] In addition, it was reported previously that Treg cell depletion in vivo drives the reappearance of Th17 cells in IL-6 signaling blockade mice.[9] Thus, it is reasonable to speculate that the blockade of IL-6 signaling in mice with EAU leads to the promotion of IRBP-specific Treg cell generation, which, in turn, suppresses IRBP-specific Th1 and Th17 response. Furthermore, because the IL-6 signaling blockade can abolish Th17 cell differentiation directly,[12] this Treg promotion should be of particular importance in the suppression of IRBP-specific Th1 cells.

We attempted to investigate the mechanisms involved in Treg cell suppression of IRBP-specific Th1 cells in vivo. Although we were able to obtain Treg cells from lymph nodes, we were not able to obtain Treg cells from the eye. In addition, because IRBP-specific Treg cells could be detected only by coculture with IRBP for a few days and intracellular staining of Foxp3 requires the fixation of cells, we could not obtain purified Treg cells as live cells. Future studies to understand more clearly the role of Treg cells in the suppression of IRBP-specific Th1 responses in vivo in EAU would be of interest.

Similar to the EAU murine model, both Th17[1,12] and Th1 cells[35–37] are considered to be involved in human uveitis and scleritis. Therefore, we speculate that the IL-6 signaling blockade, which can suppress both Th17 and Th1 responses in vivo, may show therapeutic efficacy in refractory uveitis and scleritis. Anti-human IL-6R mAb (tocilizumab) is used for the treatment of rheumatoid arthritis (RA)[38,39] and is also effective for RA patients who do not respond to anti-TNF therapy. Future clinical studies are warranted to investigate whether tocilizumab is also effective for various forms of human uveitis.

In summary, our study suggest that IL-6 is a key cytokine that determines the fate of IRBP-responsive CD4 T cells, inhibiting the generation of immunosuppressive IRBP-responsive Treg cells and promoting IRBP-specific Th17 and Th1 cells. This is likely the reason IL-6 signaling blockade completely

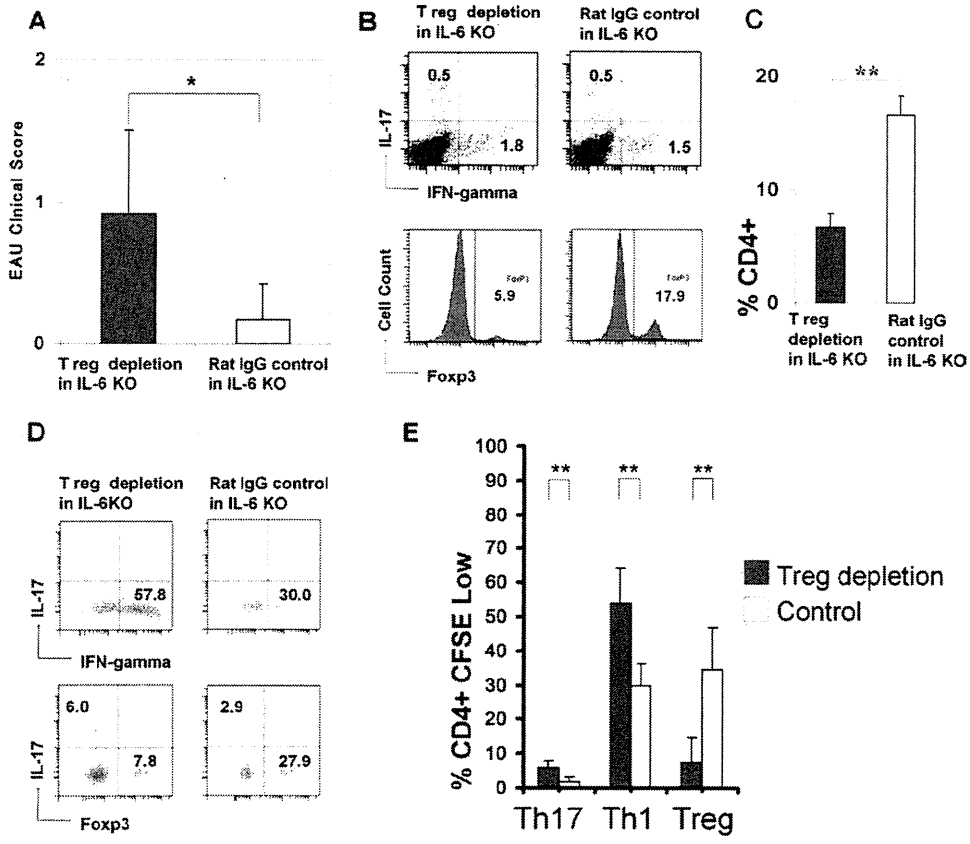

FIGURE 5. Development of EAU in Treg cell-depleted IL-6KO mice. IRBP-specific Th17 and Th1 cells increased in Treg cell-depleted IL-6KO mice. (**A**) In IL-6KO mice, Treg cell depletion induced EAU inflammation. Equal amounts of rat IgG were used as control. Data show the average ± SD of six mice. Mann-Whitney *U* test; $^{*}P < 0.05$. (**B**) Intracellular cytokine staining for CD4 and Foxp3 in lymph node cells on day 10 in vivo confirmed that Treg cells were significantly decreased in Treg cell-depleted mice; however, Th17 and Th1 cells were not changed. Values in boldface are representative data obtained from one mouse per group; all groups include at least three mice. (**C**) Frequency of $Foxp3^{+}$ Treg cells in Treg cell-depleted and control IL-6KO mice on day 10. Data show the average ± SD of three to four mice. Student's *t*-test; $^{**}P < 0.01$. (**D**) Lymph node cells on day 10 were labeled by CFSE and cocultured with IRBP, as described. $CD4^{+}$ cells were gated. Values in boldface are representative data obtained from one mouse per group; all groups include at least five mice. (**E**) Frequency of IRBP-specific Th1 and Th17 cells in Treg cell-depleted IL-6KO mice was significantly higher than that of control mice, and IRBP-specific Treg cells in Treg cell-depleted IL-6KO mice were lower than those of control mice. Data show the average ± SD of five to six mice. Student's *t*-test for the difference in frequencies of Th17, Th1, and Treg cells. $^{**}P < 0.01$.

suppresses the development of EAU in which Th1 and Th17 cells are implicated. Anti-IL-6R mAb therapy may thus be effective in either Th1- or Th17-dominant diseases such as human uveitis and may also be useful for the treatment of inflammatory disease refractory to current therapies.

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CASE REPORT

A case of Behçet's disease treated with a humanized anti-interleukin-6 receptor antibody, tocilizumab

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Abstract A 47-year-old female patient with Behçet's disease had been treated with colchicine, prednisolone, cyclosporine A, and infliximab. Because she relapsed, however, treatment with tocilizumab, a humanized anti-interleukin 6 receptor antibody, was started. This treatment suppressed the patient's clinical manifestations, including ocular attacks, for 1 year and improved her visual acuity. This experience indicates that tocilizumab may constitute a therapeutic option for refractory Behçet's disease.

Keywords Behçet's disease · IL-6 · Tocilizumab

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Introduction

Behçet's disease (BD) is a systemic inflammatory disease, characterized by recurrent oral and genital ulcers, skin and ocular lesions, and other manifestations including neurological, gastrointestinal, and vascular involvements [1]. In BD patients, recurrent ocular attacks, especially of posterior uveitis, eventually lead to irreversible visual loss at a reported frequency rate of about 25% [1]. Patients with BD with refractory central nervous system (CNS) involvement (neuro-BD) often suffer irreversible loss of cognitive function in conjunction with various neurological disturbances. These disease manifestations and the irreversible loss of function have a major negative impact on patients' quality of life (QOL), while severe involvement of the CNS, gastrointestinal tract, and blood vessels can be life-threatening, especially for adolescent male patients. Several treatment regimens using immunosuppressants such as cyclosporine A and azathioprine, interferon α (IFNα), and anti-tumor necrosis factor α (anti-TNFα) agents have been developed and their effectiveness for refractory BD has been proven [1–4]. For patients resistant to these therapeutic regimens, however, no other effective therapies are available at present.

The pathogenesis of BD is not yet fully understood, but it is known that genetic factors as well as environmental factors interact with each other, leading to the activation of immunological and inflammatory responses [1]. As for the genetic background, a higher than normal positivity for HLA-B51 is well known to be associated with the occurrence of BD, as are genetic polymorphisms in several genes, including those for interleukin-1 (IL-1); intracellular adhesion molecule-1 (ICAM-1); endothelial nitric oxide synthetase (eNOS); Mediterranean fever (MEFV), IL-10, and IL-12/IL-23 receptor β1 [1, 5, 6]. Most patients with

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BD show hypersensitivity to the oral streptococcal bacterium *Streptococcus sanguinis*. Molecular mimicry between heat shock proteins in bacteria and humans is thought to activate T cells such as Th1 and Th17 to produce distinct cytokines leading to neutrophil activation [1]. In fact, several studies have shown that serum levels of cytokines including IL-2, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IFNγ, and TNF α are elevated in patients with active BD [1, 7]. The production of IL-6 from the T cells, monocytes, and peripheral blood mononuclear cells (PBMCs) of BD patients is reportedly enhanced in vitro [8, 9]. In addition, IL-6 is the only cytokine which is elevated in the cerebrospinal fluid of patients with the chronic progressive type of neuro-BD [10, 11]. These findings indicate that IL-6 may have a pathologic role in BD and could thus be a candidate target molecule for the treatment of BD. In the study reported here, we used tocilizumab, a humanized anti-IL-6 receptor antibody [12], for a patient with BD who had been refractory to conventional therapeutic drugs.

Case report

A 35-year-old woman was diagnosed with BD in 1997 on the basis of the presence of recurrent oral and genital ulcers, erythema nodosum, and uveitis, which met the classification criteria for BD established by the Japanese Ministry of Health and Welfare [13]. Because there was no evidence of involvement of the CNS, gastrointestinal tract, or blood vessels, the patient was initially treated with colchicine and prednisolone (20 mg/day). Although cyclosporine A was added in combination with colchicine and prednisolone, the patient's severe posterior uveitis persisted and her visual acuity deteriorated (right eye from 1.2 to 0.2, left eye from 1.2 to 0.15). In August 2007, administration of infliximab, a monoclonal chimeric antibody against TNFα, was started, at a dose of 5 mg/kg, followed by a regular treatment schedule (at weeks 2 and 6, and then every 8 weeks). The infliximab treatment resulted in almost complete disappearance of the clinical manifestations, including the oral and genital ulcers, erythema nodosum, and uveitis, indicating that the disease had gone into remission. In spite of the continuous treatment with infliximab, however, the disease flared up again in December 2008, and the patient presented with recurrent oral ulcers, erythema nodosum, and uveitis, and her visual acuity in the right eye had worsened even more (from 0.2 to 0.1).

Because the disease activity could not be controlled with colchicine, prednisolone, cyclosporine A, and infliximab, we considered azathioprine or tocilizumab as a therapeutic option. The ethics committee of Osaka University Hospital approved the use of tocilizumab for this patient, and her written informed consent was obtained. Infliximab was discontinued in April 2009, and monthly intravitreal injections of triamcinolone were continued until the administration of tocilizumab was instituted, and this regimen resulted in a transient amelioration of her visual acuity. In June 2009, when the patient was 47 years old, treatment with tocilizumab (8 mg/kg, every 4 weeks) was started as monotherapy; that is, without the intravitreal injections of triamcinolone or the concomitant use of colchicine, cyclosporine A, or prednisolone. The tocilizumab was paid for with a grant from the Program for Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. The patient's serum concentration of IL-6 before the beginning of the treatment was 5.2 pg/ml (normal value <4). The clinical effect of tocilizumab was assessed by using the European Behçet's disease current activity form (BDCAF) [14], which assesses headache, oral ulceration, genital ulceration, erythema nodosum, and arthralgia on a scale of 1 to 4 (0, no symptoms; 1, symptoms for 1 week; 2, symptoms for 2 weeks; 3, symptoms for 3 weeks; 4, symptoms for 4 weeks). Change of visual acuity was also monitored. In addition, QOL was assessed with the short-form 36-item health survey, version 2 (SF-36v2) [15] before the treatment and at the 6 and 12th tocilizumab infusions. The SF-36v2 assesses physical health in terms of physical functioning (PF), role physical (RF), body pain (BP), and general health perceptions (GH), and mental health in terms of vitality (VT), social functioning (SF), role emotional (RE), and mental health (MH).

During the tocilizumab treatment, the clinical manifestations such as genital ulcers, erythema nodosum, and uveitis were attenuated and these manifestations remained silent for 12 months. Changes in the BDCAF score and visual acuity are shown in Fig. 1. The frequency and severity of ocular attacks decreased, along with improvement in visual acuity (right eye from 0.2 to 0.3, left eye from 0.4 to 0.4). Occasional administration of low-dose prednisolone (5 to 15 mg/day for 2 days) was required for mild ocular attacks, but after the 6th infusion of tocilizumab even such occasional use of oral steroids became unnecessary. The patient became entirely free of genital ulcers and erythema nodosum, although oral ulcers were observed occasionally, but they were few in number. The overall severity of her symptoms became milder than ever before. The SF-36v2 scores, especially for physical functioning, role physical, vitality, social functioning, and role emotional also improved dramatically, so that she could perform her daily living activities without difficulties (Fig. 2). The serum level of IL-6 was elevated from 5.2 to 232 pg/ml at 4 weeks after the first infusion of tocilizumab, but decreased to 22 pg/ml just before the 12th infusion.

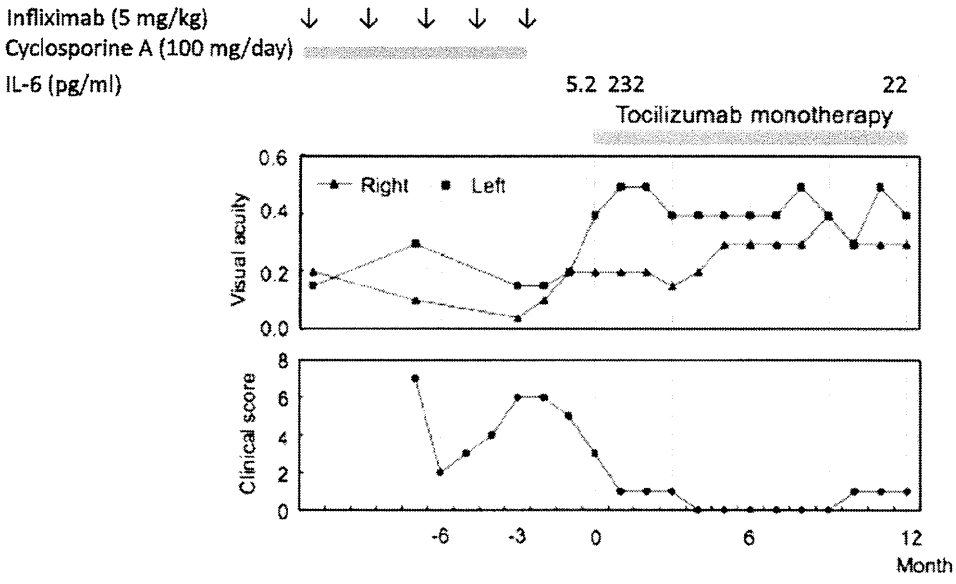

Fig. 1 Clinical effect of tocilizumab on visual acuity and Behçet's disease current activity form (BDCAF). Changes in visual acuity, summed score for headache, oral ulceration, genital ulceration, erythema nodosum, and arthralgia entered on the BDCAF, and serum interleukin-6 (*IL-6*) level are shown

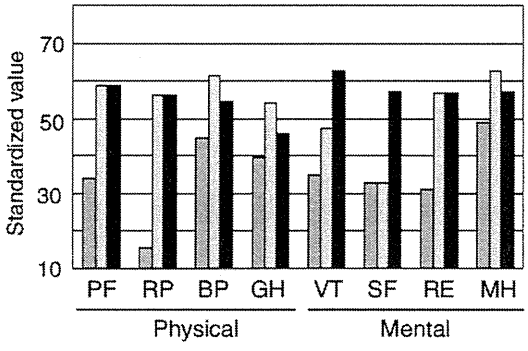

Fig. 2 Clinical effect of tocilizumab on quality of life (QOL). Changes in QOL were assessed with the short-form 36-item health survey (version 2) before tocilizumab administration (*left bars*) and at the 6th (*middle bars*), and 12th infusions (*right bars*). Standardized values (mean = 50 with standard deviation = 10) are shown. *PF* physical functioning, *RP* role physical, *BP* body pain, *GH* general health perceptions, *VT* vitality, *SF* social functioning, *RE* role emotional, *MH* mental health

There were no adverse events, except for a transient increase in the serum low-density lipoprotein (LDL)-cholesterol level (115 mg/dl at baseline, 154 mg/dl at the 6th infusion, and 123 mg/dl at the 12th infusion).

Discussion

In the case reported here, tocilizumab monotherapy for the patient resulted in a 12-month continuation of low disease activity of BD, which had relapsed during combined treatment using colchicine, prednisolone, cyclosporine A, and infliximab. The European League Against Rheumatism recommends that for refractory eye involvement in BD either cyclosporine A or infliximab should be used in combination with azathioprine and corticosteroids; alternatively IFNα with or without corticosteroids could be used [3]. Anti-TNFα agents and IFNα reportedly constitute the most potent therapies for refractory BD complicated by uveitis [1–4, 16, 17]. Infliximab for the treatment of refractory BD was approved in Japan in 2007, while IFNα has not yet been approved for this purpose. The disease activity in our patient was initially satisfactorily controlled by infliximab combined with colchicine, cyclosporine A, and prednisolone, but flared up 15 months after the start of infliximab administration. After intravitreal injections of triamcinolone, her visual acuity recovered somewhat. Tocilizumab administration resulted in further attenuation of the BD clinical manifestations, not only showing attenuation of the uveitis but also attenuating the oral ulcers and erythema nodosum. Because the effect of intravitreal triamcinolone injections is thought to be limited to ocular lesions and to last for only a few months [18], we considered that the systemic clinical improvement, as well as the improvement in QOL, observed in our patient during the 12-month tocilizumab treatment period was due to the direct effect of this agent. The serum IL-6 level depends on the balance between IL-6 production and elimination, and its level during tocilizumab treatment represents the actual endogenous production of IL-6, which correlates with the true level of disease activity [19]. The serum concentration of IL-6 in our patient at baseline was 5.2 pg/ml and appeared to be not so high. But when measured at 4 weeks after the first injection of tocilizumab, the serum IL-6 level had increased to 232 pg/ml, indicating high endogenous production of IL-6. The level decreased to 22 pg/ml just before the 12th infusion of tocilizumab, suggesting the efficacy of this agent. To the best of our knowledge, this is the first case to substantiate the efficacy of tocilizumab for the treatment of BD.