CD4⁺Foxp3⁺ T-Regulatory Cells in Noninfectious Uveitis

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Objective: To evaluate CD4⁺Foxp3⁺ (forkhead box P3) T-regulatory cell populations in patients with uveitis and to determine if T-regulatory cell populations are associated with disease features.

Methods: Patients with uveitis were evaluated for CD4⁺Foxp3⁺ T-regulatory cells by flow cytometry. Systemic and ocular diagnoses, disease activity, and the presence of cystoid macular edema were reviewed. Percentages of CD4⁺Foxp3⁺ lymphocytes were compared for patients with inactive vs active disease, systemic vs ocular diagnoses, and the presence or absence of cystoid macular edema. Real-time polymerase chain reaction testing was performed on 2 patients with extremely low CD4⁺Foxp3⁺ cell populations to assess Foxp3 mRNA.

Results: A total of 20 patients with intermediate uveitis, posterior uveitis, and panuveitis were evaluated. The mean age was 40.6 years and the mean visual acuity was 20/57. Percentages of CD4⁺Foxp3⁺ cells were lower in patients with active compared with inactive uveitis (P < .05). No differences in T-regulatory cells were observed between the other subgroups. Two patients with recalcitrant uveitis who demonstrated less than 1% CD4⁺Foxp3⁺ lymphocytes showed extremely low or absent Foxp3 mRNA.

Conclusion: T-regulatory cells are reduced in patients with active compared with inactive disease. Severe depletion of CD4⁺Foxp3⁺ T cells and Foxp3 mRNA in 2 patients with severe uveitis suggests that loss of the T-regulatory cells of uveitis may be a salient feature in certain patients.


UVEITIS HAS BEEN REPORTED TO CONTRIBUTE TO THE CAUSE OF UP TO 10% OF CASES OF BLINDNESS IN DEVELOPED NATIONS AND MAY RESULT IN SIGNIFICANT VISUAL HANDICAP IN UNTREATED PATIENTS.¹ A RECENT REPORT SUGGESTED THAT UVEITIS OCCURS WITH AN INCIDENCE OF 52 IN 100 000 PERSON-YEARS, WITH A PREVALENCE OF 115 IN 100 000 PERSONS.² BECAUSE OF THE SIGNIFICANT IMPACT OF UVEITIS ON VISUAL DISABILITY AND BLINDNESS, INVESTIGATION OF THE BASIC MECHANISMS UNDERLYING OCULAR INFLAMMATION IS WARRANTED.

Evaluation of peripheral blood and aqueous fluid from patients and studies of experimental autoimmune uveitis have supported the role of CD4⁺ T-helper type 1–mediated processes in the perpetuation of intraocular inflammation.³ The roles of CD8⁺ cells, B cells, and natural killer cells are also thought to be important, but are less clearly defined.

The role of regulatory T cells in modulating autoreactive T cells in uveitis and other autoimmune diseases including rheumatoid arthritis,⁷,⁸ systemic lupus erythematosus,⁸ sarcoidosis,⁹–¹¹ and multiple sclerosis¹²,¹³ has recently gained attention. Several studies have demonstrated alterations in immunoregulatory T cell populations in patients with uveitis.¹⁴,¹⁵ In experimental autoimmune uveitis, the adoptive transfer of T-regulatory cells has been shown to confer protection against intraocular inflammation.¹⁶,¹⁷

Several T-regulatory cell populations have been identified and are defined by cell surface markers, mechanism of action, and tissue of origin. The characterization of the transcription factor forkhead box P3 (Foxp3) as a key regulator of CD4⁺CD25⁺ T cells has been valuable in identifying a subset of T-regulatory cells.¹⁸,¹⁹ These CD4⁺CD25⁺Foxp3⁺ T cells develop in the thymus and are termed natural T-regulatory cells. This cell population is distinguished from several other classes of T-regulatory cells that develop in peripheral lymphoid organs. These cell populations, which originate in peripheral lymphoid tissues, may be Foxp3⁻ and have been termed induced T-regulatory cells because they can be generated by specific modes of antigen

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We wished to assess whether any relationships existed between CD4+/Foxp3+ T-regulatory cell populations and clinical disease characteristics in a group of patients with uveitis. We evaluated the percentages of CD4+Foxp3+ lymphocytes using a 3-color intracellular flow cytometry protocol in a group of patients from a tertiary uveitis referral center. We then examined whether differences existed between CD4+ Foxp3+ levels in various subgroups of patients with uveitis (systemic vs isolated ophthalmic diagnoses, presence or absence of cystoid macular edema, active or inactive uveitis).

In this series of patients, we observed 2 patients with extremely low intracellular CD4+/Foxp3+ expression who demonstrated severe active uveitis and were recalcitrant to a number of immunosuppressive therapies. Evaluation of Foxp3 mRNA confirmed extremely low or undetectable levels of Foxp3 transcript in peripheral blood lymphocytes in these 2 patients, and their clinical features are summarized herein.

**METHODS**

Patients were evaluated at the National Eye Institute at the Clinical Center, National Institutes of Health, under an institutional review board–approved protocol. Informed consent was obtained from all patients for ophthalmic examination and peripheral blood draws for research purposes. All research conformed to the Association for Research in Vision and Ophthalmology statement on human and animal research and the Declaration of Helsinki.

**BASELINE OPHTHALMIC EXAMINATION AND DATA COLLECTION**

Medical record data gathered from each patient evaluated included demographic information (age, race, sex), diagnosis classified by anatomic location according to the Standardization of Uveitis Nomenclature criteria,20 laterality of disease, systemic vs isolated ocular inflammatory disease, visual acuity (ie, best-corrected Snellen visual acuity or corrected Snellen visual acuity with an Early Treatment Diabetic Retinopathy Study chart at 4 m), the presence or absence of disease activity, the presence or absence of cystoid macular edema by optical coherence tomography and/or fluorescein angiography, and visual acuity with an Early Treatment Diabetic Retinopathy Study best-corrected Snellen visual acuity or corrected Snellen visual acuity with an Early Treatment Diabetic Retinopathy Study chart at 4 m).

**LYMPHOCYTE IMMUNOPHENOTYPING AND INTRACELLULAR STAINING FOR FOXP3**

Patients evaluated in this study underwent peripheral blood isolation for lymphocyte immunophenotyping and intracellular staining for Foxp3+ cells. Lymphocyte phenotyping was performed using previously described methods.21 Briefly, peripheral blood mononuclear cells were purified by Ficoll gradient centrifugation from 10 mL of peripheral blood drawn from patients and controls. Controls consisted of healthy volunteers aged 17 years and older. Most donors were aged between 30 and 55 years (information obtained from the National Institutes of Health Blood Bank).

Intracellular staining for Foxp3 and surface staining for phenotype were performed using fixation and permeabilization buffers provided by a Foxp3 phenotyping kit according to the manufacturer's instructions (ebioscience, San Diego, California). Alexa Fluor–labeled antihuman Foxp3 (clone PCH1010) was obtained from eBioscience. Patients and controls were paired for Foxp3 staining the same day to minimize staining variations. Either staining with an isotype-matched immunoglobulin control or nonstaining control was used to define negative staining and to establish a gate for Foxp3 staining. The compensation procedure was done following a standard flow cytometry multicolor staining protocol.

The following antibodies for cell surface staining were used: Phycoerythrin–Cy7–labeled CD3 (clone SK7), allophycocyanin–labeled antihuman CD8 (clone RPA-T8), and phycoerythrin–labeled antihuman CD4 (clone RPA T4). All of these were obtained from BD Pharmingen (San Diego, California).

Flow cytometry data were analyzed by FlowJo software (Tree star, San Jose, California). Briefly, lymphocytes were gated based on cell optic characteristics (forward scatter vs side scatter). Populations of CD3+, CD4+, CD3−, CD4+, CD8+, and CD8− as well as FoxP3+ and FoxP3− cells were gated based on antibody staining. A subpopulation was derived from its parent population. The gate for the positive staining of Foxp3 was set based on a negative control (above). The percentage of FoxP3+ cells in a defined subpopulation was calculated as the total number of FoxP3+ cells in the subpopulation divided by the number of cells of the parent cell population.

**REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS FOR FOXP3**

Two patients (patients 1 and 2) with low CD4+/Foxp3+ levels were assessed for Foxp3 mRNA levels with real-time polymerase chain reaction (PCR) analysis using SYBR-Green based quantitative PCR technique (SA Biosciences, Gaithersburg, Maryland). Peripheral blood mononuclear cells were isolated from whole blood as previously described.21 Total RNA samples were purified from peripheral blood mononuclear cells using the RNeasy kit (QIAGEN, Valencia, California). For real-time PCR analysis, 1 μg of each of the RNA samples from the patients with uveitis or controls were reverse transcribed into complementary (cDNA) strands using a first-strand cDNA reverse transcriptase kit (SA Biosciences). Real-time PCR was performed using an ABI 7500 real-time PCR unit (Applied Biosystems, San Mateo California). Assay controls included no template control and β-actin housekeeping gene control for normalization purposes. Primers for Foxp3 and β-actin genes were purchased from SA Biosciences and both primers had been pretested and confirmed by the manufacturer. Analysis of real-time PCR results strictly followed the manufacturer's instructions (SA Biosciences). Data analysis is based on the ΔΔCt method, with normalization of the raw data to housekeeping genes as described in the manufacturer's manual.

**STATISTICAL ANALYSIS**

The main outcome measure in this study was the percentage of CD4+ Foxp3+ lymphocytes relative to the total number of lymphocytes for each patient. This percentage was used as a continuous variable in analyses. Wilcoxon 2-sample rank sum tests (Mann-Whitney U test) were performed to determine whether statistical differences existed between the following subgroups of patients with uveitis: active vs inactive disease,
systemic autoimmune disease vs isolated ocular diagnosis, and presence vs absence of cystoid macular edema. Owing to the small sample size, the t-approximation of the Wilcoxon 2-sample test statistic was used and \( P < .05 \) were considered statistically significant. Linear regression was used to determine whether visual acuity was associated with the percentage of CD4+ Foxp3+ T-regulatory cells.

**RESULTS**

A total of 20 patients were enrolled; 15 (75%) were women, and the mean age was 40.6 years (range, 15-61 years). The mean visual acuity of the patients evaluated was 20/57. The demographic characteristics and anatomic sites of ocular inflammation are summarized in Table 1. Anatomic diagnoses included intermediate uveitis (9 patients, 45%), posterior uveitis (4 patients, 20%), and panuveitis (7 patients, 35%). Most patients (80%) were on immunomodulatory therapy at the time of their evaluation, and nearly all patients (90%) demonstrated bilateral disease. The clinical characteristics and CD4+ Foxp3+ T-regulatory cell populations of patients evaluated are summarized in Table 2.

The percentage of CD4+ Foxp3+ lymphocytes in patients with active uveitis (median, 4.3%; range, 0.56%-7.62%) was lower than in patients with inactive disease (median, 6.2%; range, 2.93%-18%; \( P = .047 \) (Table 3 and Figure 1). No statistically significant differences in the percentage of CD4+ Foxp3+ lymphocytes were observed between patients with known systemic autoimmune diseases vs isolated ocular diagnoses (median, 4.6% and 3.8%, respectively; \( P = .12 \)) or when comparing patients with cystoid macular edema with those with no evidence of cystoid macular edema (median, 5.8% and 5.4%, respectively; \( P = .43 \)).

No linear association between the percentage of CD4+ Foxp3+ lymphocytes and logarithm of the minimum angle of resolution (logMAR) visual acuity of the worse eye was observed. For each unit change in logMAR visual acuity (ie, a decrease in Snellen visual acuity from 20/20 to 20/200), the percentage of CD4+ Foxp3+ lymphocytes decreased, on average, by 0.018 (\( P = .37 \)).

Of the patients who were assessed for CD4+ Foxp3+ staining, 2 had extremely low levels of CD4+ Foxp3+ expression (patient 1, 0.95% and patient 2, 0.56% CD4+ Foxp3+ regulatory T cells). Expression of Foxp3 mRNA was tested to determine if this phenomenon could be verified at the transcript level as well. Expression levels of Foxp3 mRNA transcript in both patients were either undetectable or exceedingly low by real-time PCR and this was repeated twice per patient. In patient 1, Foxp3 expression was 13-fold lower (\( \Delta \Delta C_t, 3.76 \)) compared with a control patient (standardized with housekeeping gene \( \beta \)-actin) and in patient 2, Foxp3 expression was undetectable. Both patients displayed panuveitis and required multiple immunosuppressive agents. However, their clinical phenotypes differed in several respects and are herein described in detail.

**PATIENT 1**

Patient 1 is a 35-year-old white man with a history of Wegener granulomatosis–associated scleritis, panuveitis, cystoid macular edema, and a choroidal inflammatory lesion involving the right eye (Figure 2). He had a history of episcleritis in the left eye, but this eye had been quiescent for 2 to 3 years. His medical history was notable for glomerulonephritis and chronic sinusitis. Immunosuppressive medications at the time of his evaluation for CD4+ Foxp3+ lymphocyte populations included 125 mg of cyclosporine twice per day (approximately 2.5 mg/kg), 8 mg/kg of infliximab monthly, and 40 mg/d of prednisone. Prior immunosuppressive therapy had included etanercept (Enbrel; Amgen, Thousand Oaks, California) and 20 mg/wk of methotrexate. Ophthalmic examination revealed visual acuity of 20/160 OD and 20/20 OS. Slitlamp examination was significant for diffuse 2-3+ scleral inflammation, peripheral keratitis, 1-2+ anterior chamber cell and flare, 1+ vitritis, and a large, elevated, peripheral choroidal inflammatory lesion extending approximately 4 clock hours, with a surrounding exudative retinal detachment (Figure 2, A and B). Cystoid macular edema was also present.

Flow cytomorphometry showed an extremely low level of CD4+ Foxp3+ lymphocytes of 93 cells/\( \mu \)L, and the percentage of CD4+ Foxp3+ lymphocytes was 0.95% (Figure 2C). Real-time PCR for Foxp3 mRNA transcript revealed a 13-fold decrease in Foxp3 mRNA expression compared with a control patient (eFigure; http://www.archophthalmol.com).

The patient was subsequently treated with pulse intravenous solumedrol (1 g intravenously for 3 days) and cyclophosphamide (2 mg/kg/d) was initiated; despite these measures, the diffuse severe ocular inflammatory activity persisted and the patient’s visual acuity continued to decline. A scleral perforation and a total retinal detachment eventually developed, and an enucleation procedure was required for a blind painful eye. Pathologic evaluation revealed an occlusive retinal vasculitis with granulomatous infiltration of the ciliary body, choroid, and sclera.

### Table 1. Baseline Characteristics and Anatomic Diagnoses of Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
</tr>
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<tbody>
<tr>
<td>Total patients enrolled</td>
<td>20</td>
</tr>
<tr>
<td>Men</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Women</td>
<td>15 (75)</td>
</tr>
<tr>
<td>Mean age, y (range)</td>
<td>40.6 (15-61)</td>
</tr>
<tr>
<td>Anatomic location of disease</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>9 (45)</td>
</tr>
<tr>
<td>Posterior uveitis</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Panuveitis</td>
<td>7 (35)*</td>
</tr>
<tr>
<td>Immunomodulatory therapy</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16 (80)</td>
</tr>
<tr>
<td>No</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Laterality of disease</td>
<td></td>
</tr>
<tr>
<td>Bilateral</td>
<td>18 (90)</td>
</tr>
<tr>
<td>Unilateral</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Mean visual acuity of enrolled patients</td>
<td></td>
</tr>
<tr>
<td>All eyes</td>
<td>20/57</td>
</tr>
<tr>
<td>Better eyes</td>
<td>20/41</td>
</tr>
<tr>
<td>Worse eyes</td>
<td>20/80</td>
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</table>

*aOne patient with panuveitis (patient 1) also had active scleritis and a choroidal inflammatory lesion at the time of evaluation.
**PATIENT 2**

Patient 2 is a 25-year-old African American woman who was referred for sarcoidosis-associated panuveitis. She was treated with topical prednisolone acetate, 1%, initially but subsequently began taking 80 mg/d of prednisone. Methotrexate was subsequently initiated at a dose of 15 mg/wk, as the patient was unable to taper her prednisone regimen. She had been maintained on 20 mg/d of prednisone with recurrent vitritis and required intravitreal triamcinolone injections (40 mg) twice in the right eye and a periocular triamcinolone injection (40 mg) in the left eye. While taking prednisone, the patient reported a 100-lb (approximately 45.36 kg) weight gain. Visual acuity at the time of evaluation was 20/40 OD and 20/100 OS. Ophthalmic examination showed 2+ anterior chamber cell and flare in the right eye and 1-2+ cell and flare in the left eye. Numerous peripheral anterior synechiae were observed in both eyes. Dilated funduscopic examination showed 2+ vitritis and 2-3+ haze bilaterally with cystoid macular edema (Figure 3A).

Flow cytometry showed an extremely low level of $\text{CD}^+ \text{Foxp3}^+$ lymphocytes at 75 cells/µL, which constituted 0.56% of total lymphocytes (Figure 3B). Real-time PCR for Foxp3 transcription revealed undetectable Foxp3 mRNA expression (eFigure).

Cyclosporine therapy was recommended at a dose of 125 mg twice per day (2-3 mg/kg), with a subsequent de-
increase in anterior chamber and vitritis bilaterally at the 3-month follow-up.

**COMMENT**

In this study, we evaluated the CD4+Foxp3+ lymphocyte population in a group of patients with intermediate uveitis, posterior uveitis, and panuveitis and found that patients with active uveitis demonstrated a lower percentage of CD4+Foxp3+ lymphocytes than patients with inactive disease. We were unable to identify any differences between the percentage of CD4+Foxp3+ lymphocytes in uveitis subgroups with systemic autoimmune disease vs isolated ophthalmic diagnoses or by the presence vs absence of cystoid macular edema. We have previously observed that the percentages of CD4+/H11001 CD25/high regulatory T cells expressing Foxp3 currently is the most specific nuclear transcription factor for T-regulatory cells and has been found to express Foxp3+T cells in patients with active Behçet disease compared with patients with inactive Behçet disease and healthy control subjects.

In animal models of experimental autoimmune uveoretinitis, the adoptive transfer of CD4+/H11001 CD25/high regulatory T cells expressing Foxp3+ appears to confer protection from uveitis induced by the uveitogenic retinal antigen interphotoreceptor binding protein. However, CD8+T cells may also be stimulated by interphotoreceptor binding protein and transforming growth factor B1 to express Foxp3 in high levels and may have functionally suppressive activity. These studies suggest that, both in rodent models and in patients, there are likely a number of cellular regulatory mechanisms that may be involved in the prevention of ocular autoimmunity.

Foxp3 currently is the most specific nuclear transcription factor for T-regulatory cells and has been found to be enriched in CD4+/H11001 CD25/high T cells. However, Foxp3 expression has also been observed in CD25−/intermediate CD8+ T-cell subsets. Because the CD4+/CD25/high population does not necessarily capture all Foxp3 cells (ie, CD25/intermediate Foxp3− cells), we chose to define CD4+Foxp3+ as T-regulatory cells in this study. Several patients in our series demonstrated evidence of systemic autoimmune diseases including sarcoidosis, multiple sclerosis, Wegener granulomatosis, Behçet disease, Vogt-Koyanagi-Harada syndrome, and mixed connective tissue disease. Each of these conditions has been associated with abnormal populations of T-regulatory cells or deficits in T-regulatory suppressive function; however, we were unable to detect a difference in CD4+/Foxp3+ T-regulatory cell populations between patients with known systemic autoimmune diseases and isolated ocular diagnoses. However, the numbers of patients with each specific systemic autoimmune diagnosis were limited, and their heterogeneous nature may have contributed to this lack of a difference. Furthermore, our sample size was small, so our power to detect a difference was limited.

Patient 1, who was previously diagnosed with Wegener granulomatosis, demonstrated a particularly severe disease phenotype that featured scleritis, panuveitis, an inflammatory choroidal lesion, chronic sinusitis, and glomerulonephritis requiring multiple immunosuppressive medications. Prior studies have shown that functionally defective CD4+/CD25/high/Foxp3+ T-regulatory cells are expanded in the peripheral blood of patients with Wegener granulomatosis. However, it is possible that depletion of T-regulatory cells as well as functionally defective T-regulatory cells could be involved in conferring the severe disease phenotypes observed in Wegener granulomatosis.

**Table 3. Percentage of CD4+/Foxp3+ Cells From Total Lymphocytes**

<table>
<thead>
<tr>
<th>Clinical Variable Assessed</th>
<th>Median CD4+/Foxp3+ Cells, %</th>
<th>P Valuea</th>
</tr>
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<tbody>
<tr>
<td>Disease activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active (n=8)</td>
<td>4.3</td>
<td>.047b</td>
</tr>
<tr>
<td>Inactive (n=12)</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>Systemic diagnoses vs isolated uveitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic (n=10)</td>
<td>4.6</td>
<td>.12</td>
</tr>
<tr>
<td>Ocular (n=10)</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Cystoid macular edema</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present (n=8)</td>
<td>5.8</td>
<td>.43</td>
</tr>
<tr>
<td>Absent (n=12)</td>
<td>5.4</td>
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Abbreviation: Foxp3, forkhead box P3.

a Presence of disease activity as defined by the Standardization of Uveitis Nomenclature criteria.25

b Wilcoxon rank sum test.

**Figure 1.** Comparison of median CD4+/Foxp3+ (forkhead box P3) percentages demonstrated a lower median of CD4+/Foxp3+ T-regulatory cells in active vs inactive uveitis (P<.05).
Cystoid macular edema, which is associated with an elevation of proinflammatory cytokines and vascular endothelial growth factor, was also evaluated in our group of patients with uveitis. Our understanding of the pathogenic mechanisms underlying cystoid macular edema remains incomplete at this time; however, ischemic, inflammatory, and vascular factors likely play key roles. While it is likely that T cell–mediated mechanisms are involved with the inflammatory processes underlying uveitic macular edema, the role of defective T-regulatory cell populations in mediating cystoid macular edema was not evident from this study.

Real-time PCR evaluation of the 2 patients (patients 1 and 2) with extremely low levels of CD4+ Foxp3+ cells showed low Foxp3 mRNA transcript levels. This decrease in Foxp3 mRNA transcript levels may be due to increased mRNA degradation or altered gene expression from the population of cells analyzed. Prior studies have suggested that in rheumatoid arthritis and sarcoidosis, a reduction in peripheral blood T-regulatory cell populations in mediating cystoid macular edema was not evident from this study.

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autoimmunity. Decreased CD4+ population may play a role in the pathogenesis of ocular cye percentages between patients with active and inactive disease suggests that a deficiency in T-regulatory cell populations may play a role in the pathogenesis of ocular autoimmunity. Decreased CD4+ Foxp3+ lymphocyte percentages between patients with active and inactive disease suggests that a deficiency in T-regulatory cell populations may play a role in the pathogenesis of ocular autoimmunity. Lack of T- regulatory CD4+ cells may be salient features of some uveitic entities, this phenomenon may not be generalizable to all patients with uveitis. Whether Foxp3+ T-regulatory cell populations are depleted or functionally defective (ie, lacking suppressive T-cell function) in specific types of uveitis requires further study and may provide additional insight into the pathogenesis of ocular inflammatory disease.

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Additional Information: The eFigure is available at http://www.archophthalmol.com.

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