

# T-Lymphocyte Subset Distribution and Activity in Patients With Glaucoma

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**PURPOSE.** Besides glia-driven neuroinflammation, growing evidence from analysis of human blood samples, isolated autoantibodies, and postmortem tissues also support systemic immune responses during neurodegeneration in glaucoma patients. To explore the T-cell-mediated component of systemic immunity, this study analyzed T lymphocytes in patients' blood.

**METHODS.** Blood samples were collected from 32 patients with glaucoma and 21 nonglaucomatous controls, and mononuclear cells were isolated by Histopaque density gradient centrifugation. T-cell subset distribution was analyzed by multicolor flow cytometry after helper (Th) and cytotoxic fractions, and Th subpopulations, were stained with antibodies to CD4, CD8, or distinctive markers, such as IFN- $\gamma$  (for Th1), IL-4 (for Th2), IL-17A (for Th17), and CD25/FoxP3 (for T regulatory cells [Tregs]). In addition, proliferative activity and cytokine secretion of T cells were analyzed after *in vitro* stimulation.

**RESULTS.** Analysis of T-cell subset distribution detected a glaucoma-related shift. Despite similar frequencies of CD4+ or CD8+ T cells, or Th1, Th2, or Th17 subsets in glaucoma and control groups, glaucomatous samples exhibited a trend toward decreased frequency of CD4+ (or CD8+)/CD25+/FoxP3+ Tregs within the entire CD4+ (or CD8+) population ( $P < 0.001$ ). Furthermore, CD4+ T cells in glaucomatous samples presented a greater stimulation response (~3-fold) as characterized by increased proliferation and proinflammatory cytokine secretion ( $P < 0.05$ ).

**CONCLUSIONS.** These findings suggest that the immunity activated in glaucoma may not be counterbalanced by an efficient immune suppression. More work is encouraged to determine whether shifted T-cell homeostasis may contribute to neurodegeneration in glaucoma, and/or whether T-cell subset imbalance may serve as a biomarker of autoimmune susceptibility.

Keywords: glaucoma, lymphocyte subsets, immunoregulation

Among multiple pathogenic mechanisms proposed for neurodegeneration in glaucoma, neuroinflammation, evident both in the retina and optic nerve (head), is of increasing interest. It is widely thought that the sustained low-grade inflammation relying on reactive glia may represent an adaptive response to primary stress stimuli and/or neuron injury in glaucoma. However, a failure in the regulation of immune response pathways may shift this beneficial state toward a neurodegenerative process and promote secondary injury to neurons.<sup>1–7</sup>

Besides their roles in innate immune regulation, glial cells (possibly together with dendritic cells<sup>8</sup> and invading monocytes<sup>9</sup>) also have the potential to stimulate adaptive immunity. Based on present evidence, the neuroinflammatory microenvironment of glaucomatous tissues seems sufficient to favor glia-T-cell interaction and expanded immune responses. As evident in human glaucoma, increased expression and exposure of autoantigens due to tissue stress and injury,<sup>10</sup> glial inflammatory activation,<sup>11,12</sup> immunostimulatory tumor necrosis factor receptor (TNFR)<sup>13–16</sup> and toll-like receptor (TLR)<sup>17</sup> signaling, activation of the glial nuclear factor kappa-B (NF- $\kappa$ B)-regulated transcription of inflammatory mediators,<sup>16</sup> and assembly of

inflammasome for secretion of active cytokines<sup>16</sup> may all be considered as proper components to elicit systemic immunity. The proinflammatory cytokine imbalance is most prominent by increased expression of TNF- $\alpha$  in the glaucomatous human retina and optic nerve.<sup>13–16</sup> Parallel experimental studies have shown that in addition to being directly neurotoxic,<sup>18,19</sup> such proinflammatory cytokines may further activate glial responses, weaken the blood-brain barrier, and facilitate T-cell communication.<sup>20,21</sup> Accumulating information from gene or protein expression studies of glaucoma also demonstrates a marked upregulation of various chemotaxis and adhesion molecules needed for glia-T-cell interaction.<sup>16,22–27</sup> Additional evidence for adaptive immunity arises from the observations that the major histocompatibility complex II molecules are increased on reactive glia in human glaucoma<sup>28</sup> and animal models.<sup>29,30</sup> These observations, along with the prominent rise in stress-associated costimulatory molecules in glaucomatous tissues,<sup>20</sup> may support the enhanced ability for antigen presentation to T lymphocytes, which are potentially recruited to the eye (Tezel G, et al. *IOVS* 2008;49:ARVO E-Abstract 3699).<sup>21,31</sup> Over the past few decades, numerous studies of glaucomatous human donor eyes,<sup>14,16,17,28,32,33</sup> animal models<sup>27,31,34–36</sup> (Tezel G, et

**TABLE.** Demographics of the Study Groups and Clinical Characteristics of Glaucoma

Variables	Glaucoma	Control	P Value
<i>n</i>	32	21	
Age, y	70 ± 10	69 ± 9	0.6
Sex, female (%)	18 (56%)	11 (52%)	>0.05
Visual field mean deviation, dB	-10.16 ± 2.2	N/A	-
Duration of glaucoma, y	11.66 ± 1.9	N/A	-
IOP, mm Hg	16.16 ± 2.7	15.14 ± 1.9	0.1
No. of IOP-lowering medications	2.16 ± 0.6	N/A	-
Type of IOP-lowering medications			
α-Adrenergic agonists (%)	9 (28%)	-	-
β-Adrenergic blockers (%)	12 (38%)	-	-
Carbonic anhydrase inhibitors (%)	14 (44%)	-	-
Prostaglandin analogs (%)	16 (50%)	-	-

Data are presented as mean ± SD when applicable. The elevated intraocular pressure (IOP) in glaucoma patients (>22 mm Hg at diagnosis) was under control by topical treatment. Statistical significance was tested by a two-tailed *t*-test (for age and IOP intraocular pressure) or  $\chi^2$  test (for sex). N/A, not applicable.

al. *IOVS* 2008;49:ARVO E-Abstract 3699; Yang X, et al. *IOVS* 2007;48:ARVO E-Abstract 3285), glia or T-cell cultures,<sup>17,18,20,28,34</sup> or patients' blood samples<sup>37-41</sup> have aimed to better understand immunogenic aspects of glaucoma. Indeed, findings of these studies are supportive of adaptive immune responses, including increased titers of serum antibodies reacting to a variety of retina and optic nerve proteins in glaucoma.<sup>37-44</sup> Besides a complex repertoire of circulating autoantibodies, analysis of glaucomatous blood samples has also indicated altered pattern of proinflammatory cytokines.<sup>45,46</sup> Despite increasing information, however, the pathogenic importance of these immune responses is not yet well understood.

While there has been an ongoing debate about whether antibody responses are an outcome or a pathogenic mechanism of neurodegeneration, understanding of the T-cell-mediated component of systemic immunity in glaucoma remains even more limited. Studies of experimental models have provided data supportive of stimulated T-cell responses with neurodegenerative potential<sup>21,31,34</sup> (Tezel G, et al. *IOVS* 2008;49:ARVO E-Abstract 3699; Yang X, et al. *IOVS* 2007;48:ARVO E-Abstract 3285), and studies of blood samples from patient groups with glaucoma have detected some abnormalities in T-cell subsets<sup>47-49</sup>; however, better understanding of T-cell-mediated immunity requires additional studies of glaucoma. Since most of the data for T-cell-mediated immune responses in glaucoma have been generated in animal models, further studies of human glaucoma are particularly warranted. In moving forward, this study analyzed subset distribution of T lymphocytes in blood samples of patients with glaucoma and nonglaucomatous controls. In addition, inflammatory responses of these cells, including proliferative activity and cytokine production, were analyzed after in vitro stimulation.

## MATERIALS AND METHODS

### Study Groups

This study included 32 patients with primary open-angle glaucoma and a control group of 21 subjects without glaucoma. The glaucoma diagnosis was based on the assessment of elevated intraocular pressure (>22 mm Hg)

by applanation tonometry, glaucomatous optic disc cupping by funduscopy, glaucomatous visual field loss (with a pattern standard deviation <5%, or a glaucoma hemifield test result outside the 99% normal limits) by automated visual field testing (using a Humphrey visual field analyzer; Carl Zeiss Meditec, San Francisco, CA, USA), and open anterior chamber angles by gonioscopy. As an inclusion criterion, the stage of glaucoma (by considering both eyes) was moderate (with a mean deviation of < -12.00 dB<sup>50,51</sup>) to thereby enable the study of T-cell responses in a relatively early disease period. This criterion also aimed to eliminate potential effects of repeated and complicated surgical interventions (such as trabeculectomy or tube shunt insertion that may create a prolonged stimulus to induce immune responses), which are commonly applied in more advanced stages. There was no clinical evidence for alternative causes of optic neuropathy in any of the patients with glaucoma. Recruited control subjects had no clinical evidence of glaucoma, or a family history of glaucoma.

As an attempt to minimize the effects of heterogeneity between individual participants, one of the selection criteria was based on demographics, such as a similar age and sex distribution (see Table). The eyes with an ocular inflammatory disease or uveitis were not included in the study groups. Additional selection criteria were related to coexisting systemic conditions. Glaucoma patients or control subjects with an inflammatory neurodegenerative or connective tissue disease, or a history of ongoing cancer, were excluded from the study in order to eliminate potential effects of such diseases or their treatment on T-cell responses. In addition, none of the study participants had any signs of infection at the time of blood sampling or during the previous 4 weeks, and none of them had undergone any surgery during the previous 1 year.

### Sample Collection

Fasting blood samples were collected after a written informed consent was obtained according to the protocols approved by the Columbia University Institutional Review Board, and all procedures were conducted in accordance with the tenets of the Declaration of Helsinki. Approximately 10 mL venous blood was collected from each participant into a vacutainer mononuclear cell preparation tube (Becton Dickinson, Franklin Lanes, NJ, USA) containing EDTA and processed immediately. All analyses described below were run in individual samples.

### Analysis of T Cells

Mononuclear cells were isolated from blood samples by Histopaque (Sigma-Aldrich Corp., St. Louis, MO, USA) density gradient centrifugation. The isolated cells were immediately analyzed for T-cell subset distribution. In addition, cells were prepared for in vitro stimulation and expansion to subsequently analyze T-cell proliferation and cytokine secretion.

First, for immediate analysis of T-cell subsets by multicolor flow cytometry, isolated cells were stained with fluorochrome-conjugated antibodies. To define subpopulations of T cells and calculate their percentage distribution, the antibodies used for immunostaining included those reacting with distinctive subset markers as described later below under methodology for flow cytometry.

Next, for in vitro assays of inflammatory activity in individual samples, cells were resuspended at  $1.0 \times 10^6$  cells/mL in advanced RPMI medium 1640 supplemented with 2% inactivated human serum, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin (ThermoFisher Scientific, Waltham,

MA, USA). Cultured cells were stimulated with uniform magnetic beads (Dynabeads human T-activator; ThermoFisher Scientific) that were covalently coupled with antibodies to  $\epsilon$  chain of human CD3 (a subunit of the T-cell receptor complex), human CD28 (a costimulatory molecule and receptor for CD80 and CD86), and human CD137 (a costimulatory molecule expressed by activated T cells). We followed the manufacturer's instructions. Briefly, prewashed and resuspended beads (20  $\mu$ L) were incubated with a bead-to-cell ratio of 1:1 in 96-well plates placed in a humidified CO<sub>2</sub> incubator at 37°C. To expand CD4<sup>+</sup> T cells, rIL-2 (50 U/mL) was added to the incubation medium (ThermoFisher Scientific). The cell density exceeded  $2 \times 10^6$  cells/mL within 7 to 10 days after in vitro stimulation, until when the culture medium including the fresh cytokine was changed every 2 to 3 days. In vitro stimulation of T cells was also tested in the presence of retinal antigens (1  $\mu$ g/mL) that were isolated from glaucomatous or nonglaucomatous human donors (previously documented<sup>52</sup>).

### Flow Cytometry

To phenotype helper (Th) and cytotoxic fractions of T cells, isolated cells were stained with antibodies to CD4 (catalog no. 11-0048-41 and 17-0048-42; ThermoFisher Scientific/eBioscience) or CD8 (catalog no. 11-0086-41), respectively. To also analyze different subtypes of Th cells, immunostaining used additional antibodies (all from ThermoFisher Scientific/eBioscience) to distinctive markers, including IFN- $\gamma$  (catalog no. 11-7319-81) for Th1, IL-4 (catalog no. 25-7049-82) for Th2, IL-17A (catalog no. 11-7179-81) for Th17, or CD25 (also known as IL-2R $\alpha$ , catalog no. 17-0259-41) and FoxP3 (forkhead box P3, a member of the forkhead/winged-helix family of transcription factors; catalog no. 12-4776-41) for T regulatory cells (Tregs). Briefly, using a human regulatory T-cell staining kit (ThermoFisher Scientific/eBioscience), cells were resuspended in staining buffer containing a human Fc receptor-binding inhibitor at 4°C for 30 minutes. After this blocking step followed by washing, fluorescein isothiocyanate (FITC), or allophycocyanin (APC)-conjugated antibodies to surface molecules, including CD4 (6 ng/mL), CD8 (6 ng/mL), or CD25 (0.125  $\mu$ g/mL), were added for incubation at 4°C for 30 minutes. For cytoplasmic staining, cells were first permeabilized at room temperature for 60 minutes followed by a similar antibody incubation at 4°C for 30 minutes in the dark. These antibodies included FITC-conjugated antibodies (to IFN- $\gamma$ , 0.125  $\mu$ g/mL; or IL-17A, 0.125  $\mu$ g/mL), or phycoerythrin-conjugated antibodies (to IL-4, 0.1  $\mu$ g/mL; or FoxP3, 25 ng/mL). Fluorochrome-matched negative isotype antibodies were used as control. Data were acquired with LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed by using FCS express 6 software (De Novo Software, Glendale, CA, USA). A total of  $1 \times 10^4$  cells were detected in the analysis gate for each sample. Lymphocytes were gated by using forward and side scatter profiles, and the percentage of cells positive for specific markers was assessed by using two different dot-plot diagrams obtained consecutively (coefficient of variation was less than 0.20).

In addition to T-cell subset markers, multicolor flow cytometry-based analysis determined T-cell proliferation after in vitro stimulation. To quantitatively analyze newly synthesized DNA, we used a Click-iT EdU cell proliferation assay (ThermoFisher), in which the modified nucleoside EdU (5-ethynyl-2'-deoxyuridine) is incorporated during DNA synthesis for detection by a quick click chemistry reaction.<sup>53</sup> This methodology also allowed to multiplex with the fluorescent protein analysis of CD4 by flow cytometry (using CD4-FITC and EdU-APC).

### Enzyme-Linked Immunosorbent Assay

Total protein concentration was determined by a colorimetric analysis based on the Bradford protein assay (BioRad, Hercules, CA, USA). A Multi-Analyte ELISArray kit (Qiagen, Valencia, CA, USA) was used for simultaneous profiling of 12 proteins (cytokines and chemokines) in culture supernatant as previously described.<sup>54</sup> Detection sensitivity of this multiplexed cytokine assay was in the low pg/mL range. All analyses included triplicated wells and negative controls. Positive and nonspecific binding controls were provided by the kit. All concentrations were calculated from a standard curve and normalized to protein concentration in individual samples.

### Statistical Analysis

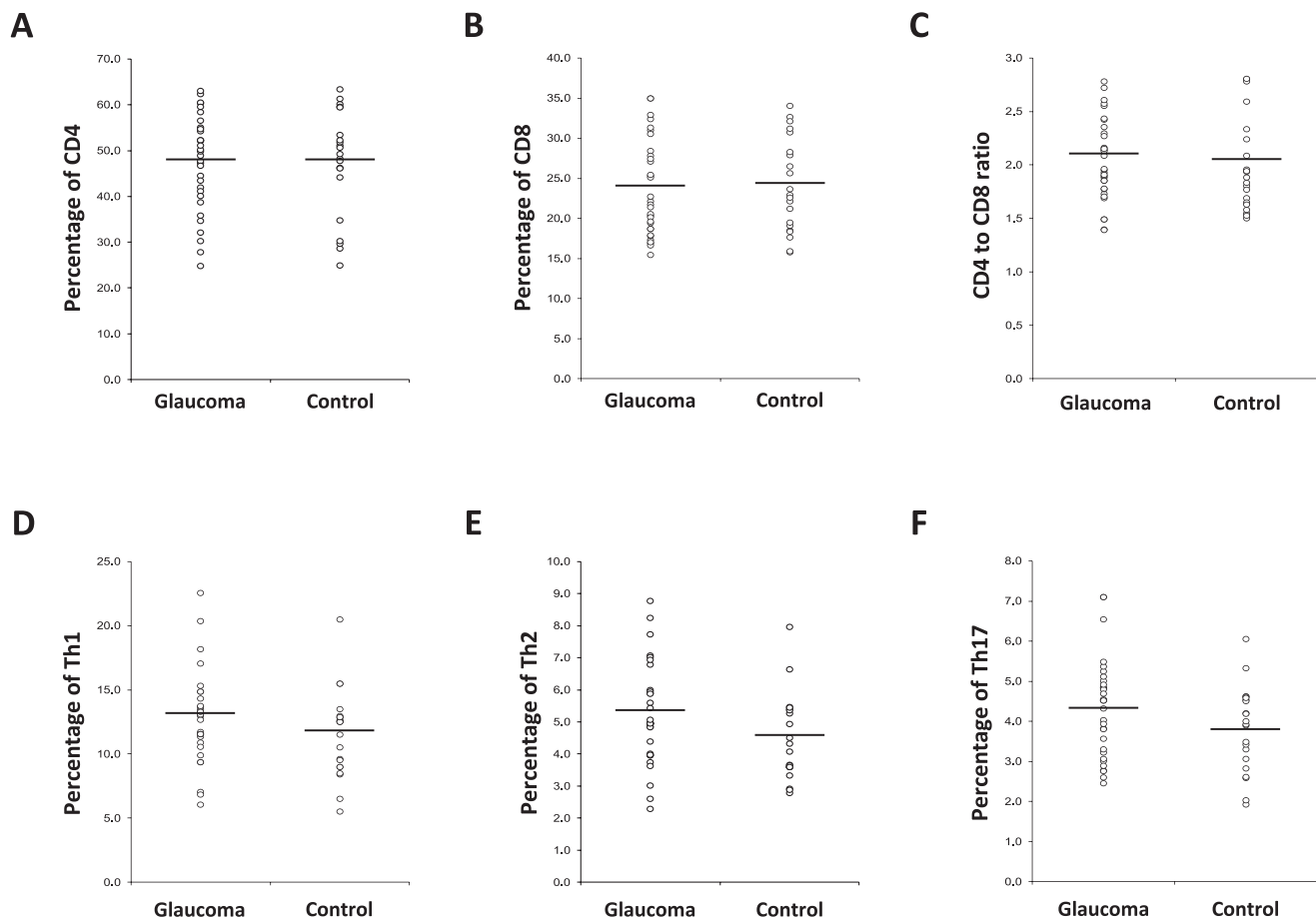
The statistical analysis was carried out by using a software package (SigmaPlot, version 12.5; Systat Software, Inc., San Jose, CA, USA). All data are presented as mean  $\pm$  SD, and bar graphs are accompanied by univariate scatterplots. A two-tailed *t*-test and  $\chi^2$  test were used for comparison of demographics and clinical characteristics between glaucoma and control groups. T-cell data were analyzed by 1-way analysis of variance (ANOVA), or Kruskal-Wallis 1-way ANOVA on ranks with Dunn's test. In addition, linear regression analysis was used to determine the relationship between T-cell subset distribution and in vitro activity. A *P* value of less than 0.05 was considered statistically significant.

### RESULTS

Demographic features of the study groups and clinical characteristics of glaucoma are presented in the Table. The mean ( $\pm$ SD) age in the glaucoma group (70  $\pm$  10 years) was not statistically different (*P* > 0.05) from that of the control group (69  $\pm$  9 years). Similarly, there was no sex difference between the two study groups (56% vs. 52% female in glaucoma and control groups, respectively; *P* > 0.05). For patients in the glaucoma group, disease duration was 11.66  $\pm$  1.9 years, and disease stage was moderate (mean deviation, -10.16  $\pm$  2.2 dB). As also shown in the Table, intraocular pressure levels were not different between glaucoma and control groups (16.16  $\pm$  2.7 mm Hg vs. 15.14  $\pm$  1.9 mm Hg; *P* > 0.05), because the elevated intraocular pressure at diagnosis of glaucoma (>22 mm Hg) was under control by treatment. As detailed in the Table, at the time of blood sampling, glaucoma patients were using a combination of topical intraocular pressure-lowering medications (2.16  $\pm$  0.6 eye drops), which included  $\alpha$ -adrenergic agonists,  $\beta$ -adrenergic blockers, carbonic anhydrase inhibitors, or prostaglandin analogs.

The mononuclear cells isolated from peripheral blood samples were immunostained for analysis of T-cell subsets by multicolor flow cytometry, and their percentage distribution was calculated in glaucomatous and nonglaucomatous samples. In addition to antibodies for CD4 and CD8, immunostaining used antibodies for distinctive markers of T-cell subsets, such as IFN- $\gamma$  for Th1, IL-4 for Th2, IL-17 for Th17, or CD25 and FoxP3 for Tregs. Percentage of CD4<sup>+</sup> (47.46%  $\pm$  10.42% vs. 47.29%  $\pm$  11.52%; *P* = 0.96) or CD8<sup>+</sup> (23.71%  $\pm$  5.95% vs. 24.10%  $\pm$  5.82%; *P* = 0.75) T cells was similar between glaucoma and control groups. When the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells was calculated (because the ratio may better reflect the outcome), it was also found to be similar in glaucoma and control groups (2.04%  $\pm$  0.38% vs. 1.99%  $\pm$  0.44%; *P* = 0.49). Although some statistically insignificant increase was noticeable in Th1 (12.83%  $\pm$  3.51% vs. 11.52%  $\pm$  3.38%; *P* = 0.09) and Th2 frequencies (5.23%  $\pm$  1.56% vs. 4.43%  $\pm$  1.32%; *P* = 0.06) in glaucomatous samples compared





**FIGURE 1.** T-lymphocyte subset distribution in glaucoma. Mononuclear cells isolated from peripheral blood samples from glaucoma ( $n = 32$ ) and control ( $n = 21$ ) groups were analyzed by multicolor flow cytometry for T-cell subset markers. (A–C) The percentage of CD4+ and CD8+ T cells and the ratio of CD4+ to CD8+ T cells were similar in glaucomatous samples and nonglaucomatous controls (ANOVA,  $P = 0.96$ ,  $P = 0.75$ ,  $P = 0.49$ , respectively). (D–F) The percentage of T-cell subsets, including CD4+/IFN- $\gamma$ + Th1, CD4+/IL-4 Th2, and CD4+/IL-17A+ Th17 subpopulations, was also similar in glaucoma and control groups (ANOVA,  $P = 0.09$ ,  $P = 0.06$ ,  $P = 0.10$ , respectively). Bars on univariate scatterplots represent the group mean.

to controls, the Th1 to Th2 ratio was similar between glaucoma and control groups ( $P = 0.44$ ). Likewise, the percentage of Th17A+ T-cell subsets ( $4.33\% \pm 1.22\%$  vs.  $3.79\% \pm 1.06\%$ ) was not different between the two groups ( $P = 0.10$ ). Related data are presented in Figure 1.

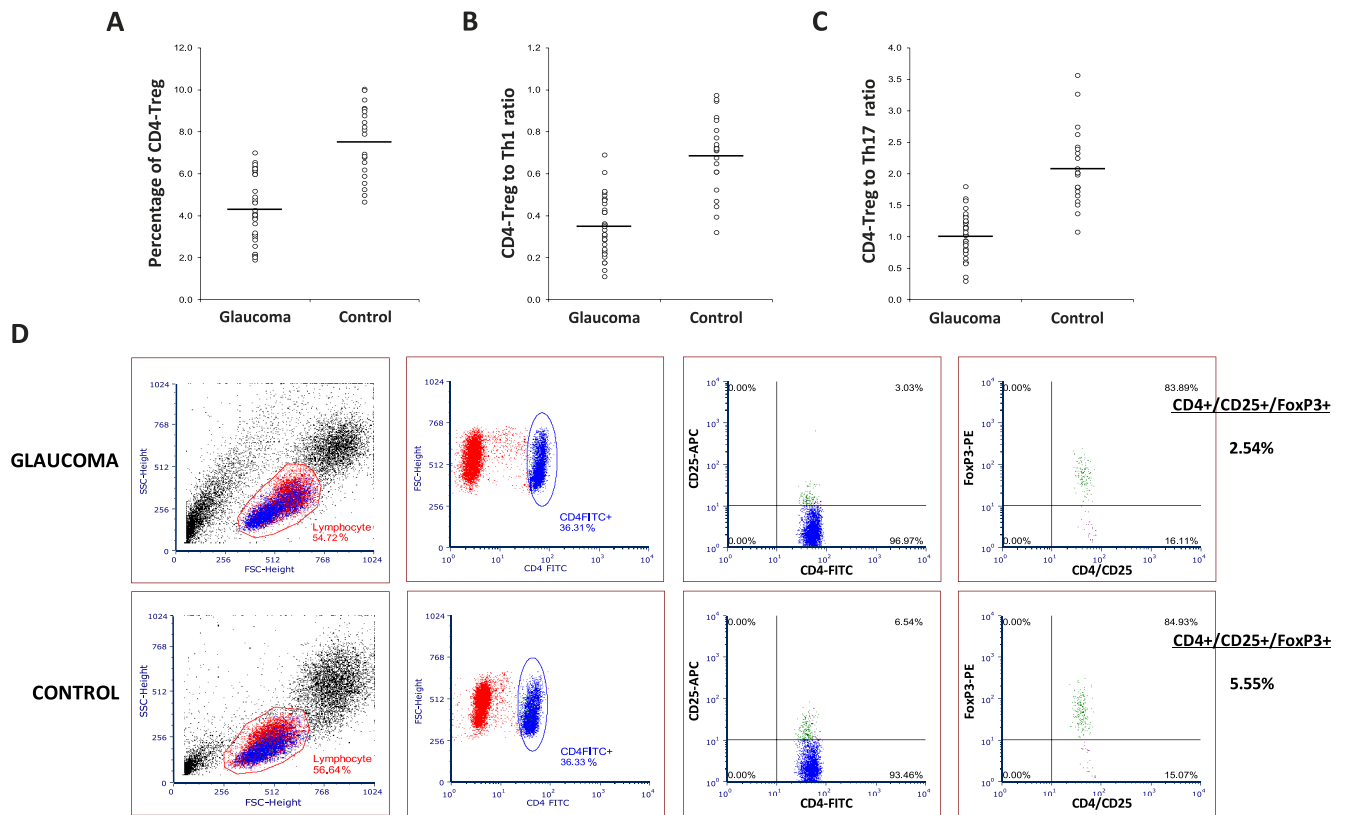
Despite a similar distribution of CD4+ or CD8+ T cells, or Th1, Th2, or Th17 subsets in glaucomatous and nonglaucomatous samples, glaucomatous samples exhibited a trend toward altered frequency of Treg subsets. As presented in Figure 2, this glaucoma-related shift was detectable by a significantly lower percentage of CD4+/CD25+/FoxP3+ Tregs within the entire CD4+ T-cell population ( $4.27\% \pm 1.56\%$  vs.  $7.51\% \pm 1.69\%$ ;  $P < 0.001$ ). The Treg imbalance was also detectable with lower ratios of CD4+ Tregs to Th1 ( $0.35 \pm 0.14$  vs.  $0.69 \pm 0.18$ ) and CD4+ Tregs to Th17 ( $1.02 \pm 0.36$  vs.  $2.09 \pm 0.61$ ) in the glaucoma group than control ( $P < 0.001$ ,  $P < 0.001$ , respectively). As presented in Figure 3, in addition to CD4+ Tregs, the percentage of CD8+/CD25+/FoxP3+ Tregs was also lower in the glaucoma group than control ( $0.75\% \pm 0.35\%$  vs.  $1.24\% \pm 0.40\%$ ;  $P < 0.001$ ). No relationship was found between the T-cell subset distribution and patients' age, sex, visual field defect, intraocular pressure, or the number or type of intraocular pressure-lowering treatment ( $P > 0.05$ ).

Next, *in vitro* assays examined the proliferative activity after *in vitro* stimulation of T lymphocytes. Compared to nonglaucomatous control samples, CD4+ T cells in glaucomatous

samples exhibited approximately 3-fold greater proliferation rate *in vitro* ( $18.48 \pm 6.49$  vs.  $6.94 \pm 2.47$ ;  $P < 0.001$ ), as assessed by flow cytometry-based analysis of EdU. Related data are presented in Figure 4.

*In vitro* analyses also included the proliferation of CD4+ T cells tested in the presence of retinal antigens isolated from glaucomatous or nonglaucomatous donors. As presented in Figure 5, T cells obtained from glaucomatous patients' blood presented a greater rate of proliferation after stimulation with glaucomatous human retinal antigens than after stimulation with nonglaucomatous retinal antigens ( $33.92 \pm 9.78$  vs.  $18.15 \pm 6.33$ ;  $P = 0.04$ ). This proliferative stimulation of glaucomatous T cells with glaucomatous retinal antigens was also much higher than stimulation of control T cells with the same antigens ( $P = 0.002$ ).

In addition, to analyze the cytokine secretion profile, a set of cytokines and chemokines was measured in culture supernatant by a multianalyte ELISArray (Qiagen). Based on this assay, the greater *in vitro* stimulation response of T cells in the glaucoma group was also evident by increased *in vitro* secretion of cytokines (Fig. 6). Compared to control samples, cytokines exhibiting a significantly increased secretion in glaucomatous samples included IL-1 $\beta$  ( $P = 0.005$ ), IL-6 ( $P = 0.03$ ), IFN- $\gamma$  ( $P < 0.001$ ), TNF- $\alpha$  ( $P = 0.003$ ), and TGF- $\beta$ 1 ( $P = 0.048$ ). Titers of chemokines, including MCP1/CCL-2 ( $P = 0.001$ ), MIP-1 $\alpha$ /CCL-3 ( $P < 0.001$ ), and MIP-1 $\beta$ /CCL-4 ( $P <$



**FIGURE 2.** T-lymphocyte subset distribution in glaucoma. Mononuclear cells isolated from peripheral blood samples from glaucoma ( $n = 32$ ) and control ( $n = 21$ ) groups were analyzed by multicolor flow cytometry for T-cell subset markers. (A) The percentage of CD4+/CD25+/FoxP3+ Tregs (CD4-Tregs) within the entire CD4+ T-cell population was significantly lower in glaucomatous samples than nonglaucomatous controls (ANOVA,  $P < 0.001$ ). (B, C) The Treg to Th1 or Treg to Th17 ratios were also significantly lower in the glaucoma group than control (ANOVA,  $P < 0.001$ ). Bars on univariate scatterplots represent the group mean. (D) Shown are representative flow cytometry images after CD4/CD25/FoxP3 immunostaining for Tregs.

0.001), were also greater in the culture supernatant of glaucomatous cells (Qiagen) than controls.

As shown by scatterplots in Figure 7, linear regression analysis detected a relationship between the frequency of CD4+/CD25+/FoxP3+ Tregs and in vitro activity of CD4+ T cells in glaucomatous samples. Many of the samples with a lower percentage of CD4+/CD25+/FoxP3+ Tregs in blood samples exhibited higher rate of proliferation ( $R = 0.670$ ,  $P < 0.001$ ), or higher titers of secreted proinflammatory cytokines ( $R = 0.516$ ,  $P = 0.002$ ;  $R = 0.725$ ,  $P < 0.001$ ; and  $R = 0.610$ ,  $P < 0.001$  for IL-1 $\beta$ , IFN- $\gamma$ , and TNF- $\alpha$ , respectively) or chemokines ( $R = 0.459$ ,  $P = 0.008$ ;  $R = 0.487$ ,  $P = 0.005$ ; and  $R = 0.666$ ,  $P < 0.001$  for MCP1, MIP-1 $\alpha$ , and MIP-1 $\beta$ , respectively) in culture.

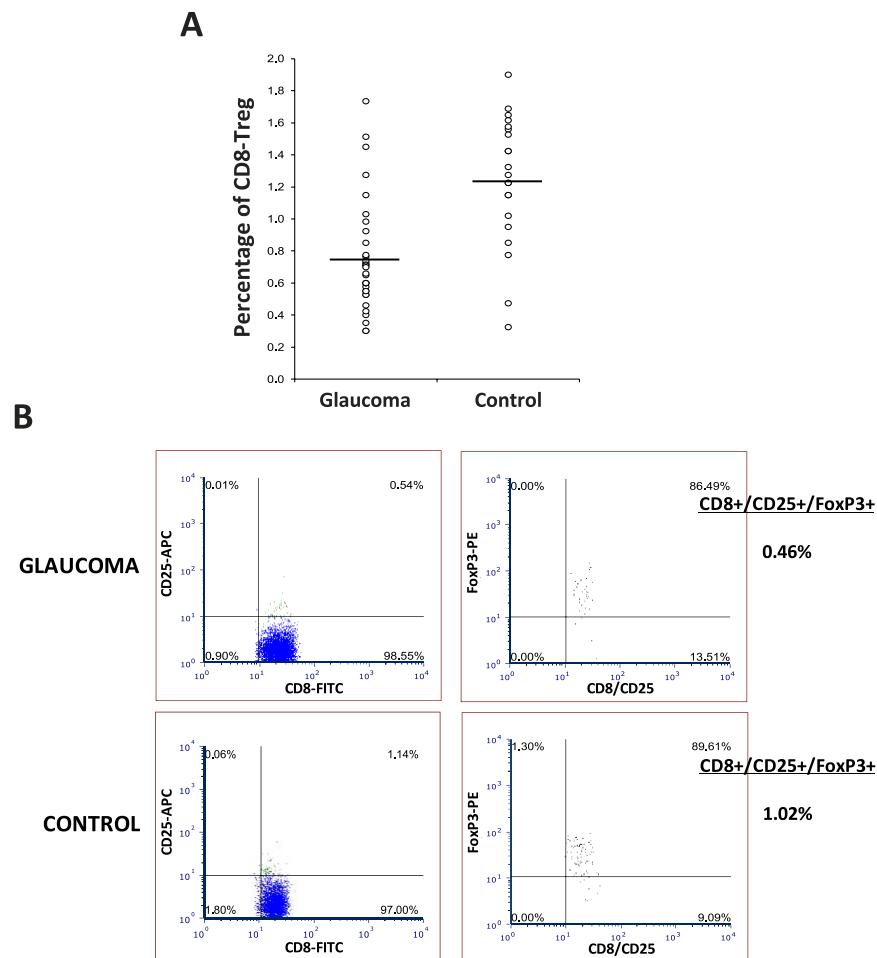
## DISCUSSION

By examining the immune fluorescent staining of distinctive markers for T-cell subsets, this study detected an imbalance of CD4+/CD25+/FoxP3+ Tregs (and CD8+/CD25+/FoxP3+ Tregs) in blood samples from patients with glaucoma compared to nonglaucomatous controls. In addition, in vitro assays detected a greater inflammatory stimulation of CD4+ T cells in glaucomatous samples than controls. As discussed later below, respecting the ability of Tregs to suppress autoimmunity and maintain immune homeostasis,<sup>55,56</sup> findings of this study suggest that the systemic immune responses may not be counterbalanced by an efficient immune suppression in glaucoma. It remains unclear, however, whether this imbalance reflects an adaptive immune response to lessen self-tolerance

and promote reparative tissue cleaning at the immune-privileged tissues (as retina and optic nerve) in glaucomatous eyes. An alternative view of whether dysregulated immune responses in glaucoma may lead to T-cell-mediated injury to neurons also remains to be further studied.

The immune system maintains its balance by contrasting activity of different T-cell subpopulations, pathogenic or suppressive, which are regulated by cytokines. Different cytokine profiles of T cells are therefore commonly used to define their functional subsets.<sup>57</sup> The CD4+ T-cell subsets are critical for immune homeostasis and host defense, but are also indicated as being a major contributor of pathology in autoimmune and inflammatory diseases, including neurodegenerative diseases.<sup>58</sup> In general, Th1 is known to mediate inflammation, Th2 stimulates B-cell proliferation and antibody production, and Th17 plays a major role in autoimmune inflammatory diseases. Tregs, in contrast, are important to maintain self-tolerance, and prevent autoimmunity.<sup>59–61</sup> Similar to CD4 Tregs, CD8 Tregs have immunosuppressive functions by secreting various inhibitory cytokines and chemokines. While CD8+ T cells demonstrate cytotoxic effects, CD8 Tregs can effectively block the overreacting immune response to maintain immune homeostasis.<sup>62–64</sup> Both CD4+ and CD8+ Tregs display decreased number and/or function in several autoimmune diseases and have been suggested as immunotherapy targets.

The present study of glaucoma found no prominent difference in distribution of IFN- $\gamma$ + (Th1), IL-4+ (Th2), or IL-17A+ (Th17) subsets of T lymphocytes, but detected lower

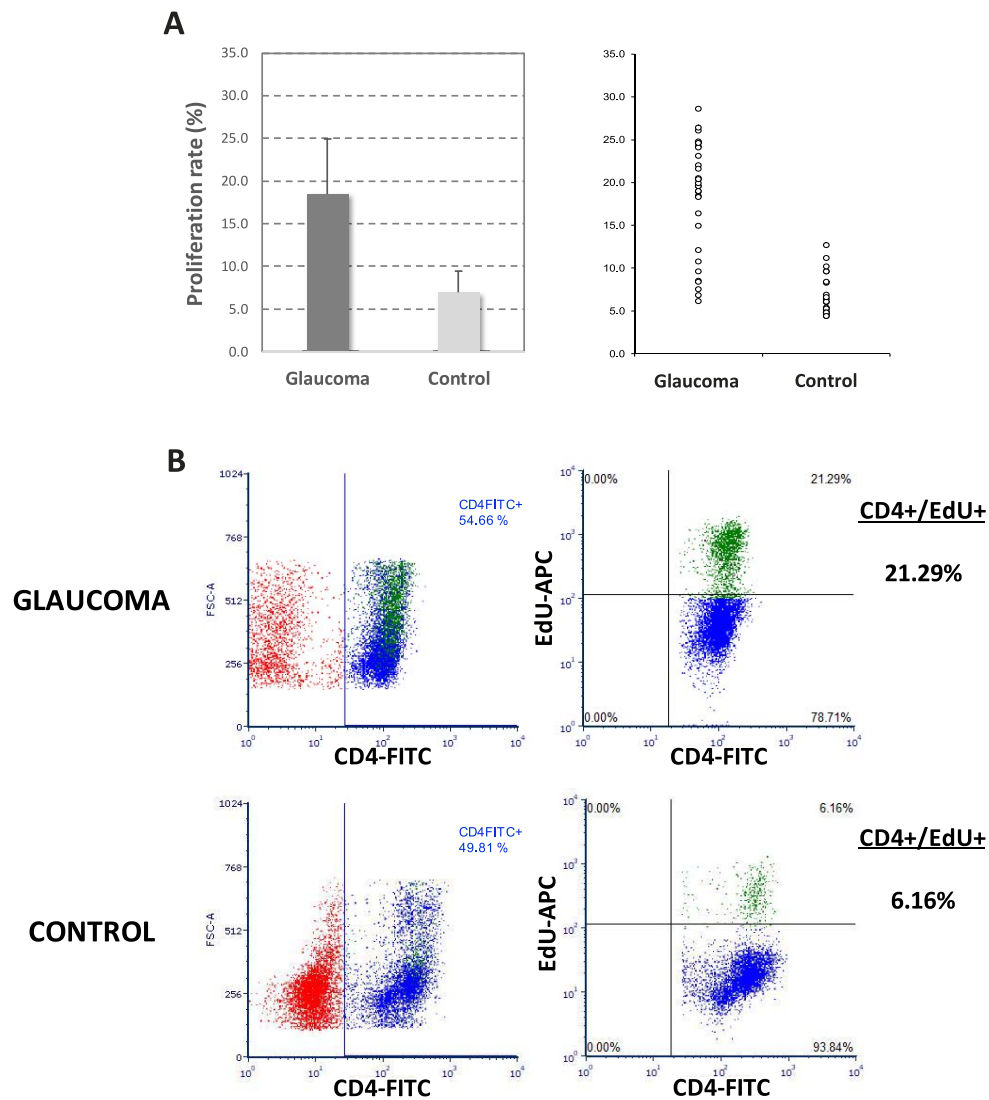


**FIGURE 3.** T-lymphocyte subset distribution in glaucoma. Mononuclear cells isolated from peripheral blood samples from glaucoma ( $n = 32$ ) and control ( $n = 21$ ) groups were analyzed by multicolor flow cytometry for T-cell subset markers. (A) Similar to CD4<sup>+</sup> Tregs, the percentage of CD8<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> Tregs (CD8-Tregs) was also significantly lower in glaucomatous samples than nonglaucomatous controls (ANOVA,  $P < 0.001$ ). (B) Shown are representative flow cytometry images after CD8/CD25/FoxP3 immunostaining for Tregs.

frequency of CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> and CD8<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> Tregs in glaucomatous samples than nonglaucomatous controls. It is notable that the immune system presents enormous complexity through molecular antagonism and plasticity of inflammatory and regulatory T-cell programs. A dynamic expression pattern of cytokines under steady-state or disease conditions may switch, or self-limit, inflammation. Besides a dominant cytokine environment, presented antigens, costimulatory molecules, and signaling cascades are also important to control the type of immune response. Multiple factors might similarly affect the T-cell subset profile in glaucoma.

Previous studies of T lymphocytes in glaucoma patients have documented altered gene expression and function.<sup>65–67</sup> An earlier study of T-cell subsets (not including Tregs) in blood samples has reported some differences between patients with primary open-angle glaucoma or normal-pressure glaucoma.<sup>47</sup> Another study<sup>48</sup> has detected a greater ratio of CD4<sup>+</sup>/CD25<sup>+</sup> Tregs (not tested for FoxP3 staining) in a small group of patients with glaucoma relative to controls. More recently, the frequency of Tregs has been found to be different between patients with primary open-angle glaucoma versus normal-pressure glaucoma, despite comparable frequencies of Th1 and Th2. Even though distinction of such glaucoma subgroups is arbitrary, and abnormal immune activity may be evident in glaucoma patients independently of their intraocular pressure, findings of the latter study support lower Treg frequency

among glaucoma patients.<sup>49</sup> Similarly, the present study of T cells detected lower frequency of CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> Tregs in the glaucoma group. In addition to T-cell subset distribution, the present study also analyzed the inflammatory response of T lymphocytes after in vitro stimulation. This analysis, despite the observational study design, allowed the search for a link between the altered Treg frequency and inflammatory responses of T cells. There was an inverse relationship between the frequency of CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> Tregs in blood samples and the rate of proliferation and proinflammatory cytokine secretion of CD4<sup>+</sup> T cells in culture. Cultured cells were not studied for T-cell subset distribution; however, the proinflammatory shift in cytokine balance toward Th1 dominance (there was a significant increase in proinflammatory Th1 cytokines, including IL-1 $\beta$ , IFN- $\gamma$ , and TNF- $\alpha$ , with no increase in IL-4 related to Th2 response) may also support the lack of efficient Treg suppression (there was some increase in TGF- $\beta$ 1 with no increase in IL-10 related to Treg response) in glaucomatous samples. Interestingly, this in vitro analysis also indicated a more prominent stimulation of T cells obtained from glaucomatous patients' blood with glaucomatous human retinal antigens (compared to nonglaucomatous retinal antigens). This observation may support increased antigenicity of the glaucomatous retina and/or increased T-cell reactivity to retinal antigens in glaucoma patients. These observations warrant further studies to determine the importance of T-cell



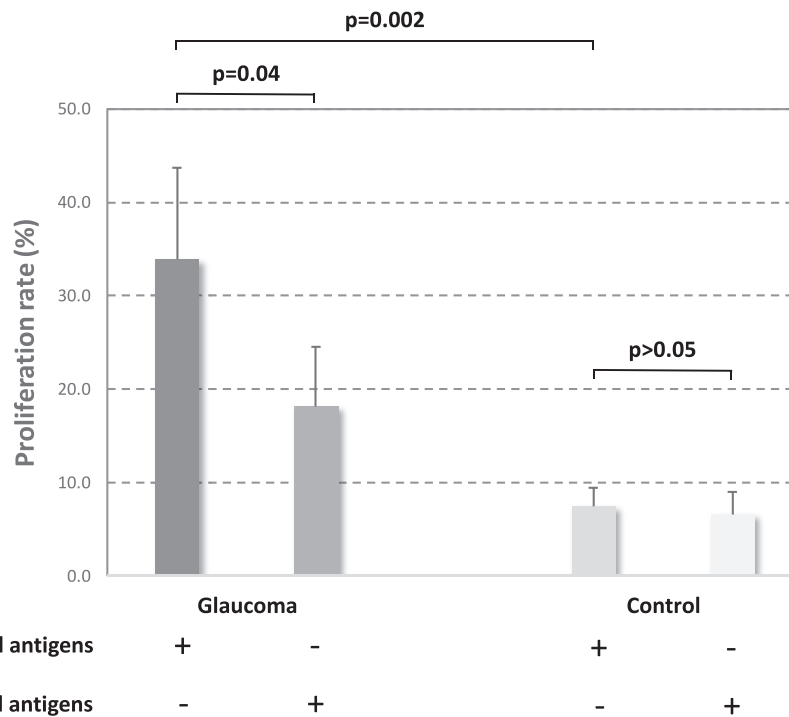
**FIGURE 4.** In vitro analysis of T lymphocytes in glaucoma. Mononuclear cells isolated from peripheral blood samples from glaucoma ( $n = 32$ ) and control ( $n = 21$ ) groups were analyzed for a proliferation marker after in vitro stimulation. **(A)** The percentage of CD4+/Edu+ cells analyzed by multicolor flow cytometry was significantly higher in glaucomatous samples than nonglaucomatous controls (ANOVA,  $P < 0.001$ ). Data are presented as mean  $\pm$  SD in bar graphs, while univariate scatterplots present data distribution. **(B)** Shown are representative flow cytometry images after triple immunostaining.

responses for neuroinflammation and neurodegeneration in glaucoma.

With respect to suppressive functions of Tregs,<sup>55,56</sup> decreased Treg frequency in glaucoma may be considered supportive of disrupted immune homeostasis. A similar Treg shift has also been observed in several other neurodegenerative diseases, in which a number of molecules, including some hormones, neuropeptides, neurotransmitters, and ion channels, have been suggested to affect this dysregulation.<sup>68,69</sup> Furthermore, recent experimental studies have indicated a beneficial role of boosting the Treg response to slow down the progression of neurodegeneration in Alzheimer's disease,<sup>70,71</sup> or ischemic stroke.<sup>72</sup> Antigenic vaccination has also been suggested to boost protective T-cell responses for neuroprotection in glaucoma<sup>73-75</sup>; however, new studies aim to transfer purified induced Tregs.<sup>76</sup>

The presented findings of T-lymphocyte analysis in glaucoma patients may also support the relevance of recent experimental findings to human disease and encourage experimental studies to develop immunomodulatory treat-

ments. Recent efforts exploring the immunogenic aspects of glaucomatous neurodegeneration have included the work to produce immune-mediated animal models. For example, immunization of rats with heat shock proteins has induced retinal ganglion cell loss in a topographically specific pattern resembling glaucoma in humans.<sup>35</sup> Subsequent immunization studies<sup>36,37</sup> have yielded similar results. Studies of transgenic mice lacking mature lymphocytes have reported some neuroprotection against ocular hypertension-induced injury (McKinnon SJ, et al. *IOVS* 2010;51:ARVO E-Abstract 2523). Moreover, T cells obtained from experimental glaucoma models have exhibited stimulated proliferative activity (Yang X, et al. *IOVS* 2007;48:ARVO E-Abstract 3285), and adoptive transfer of T lymphocytes obtained from experimental or genetic models of glaucoma into naïve animals has resulted in interaction of transferred cells with recipient microglia and provoked retinal ganglion cell loss despite the lack of intraocular pressure elevation (Tezel G, et al. *IOVS* 2008;49:ARVO E-Abstract 3699).<sup>31,77</sup> Findings of a more recent study of adoptive transfer in mice deficient for T and/or B cells have

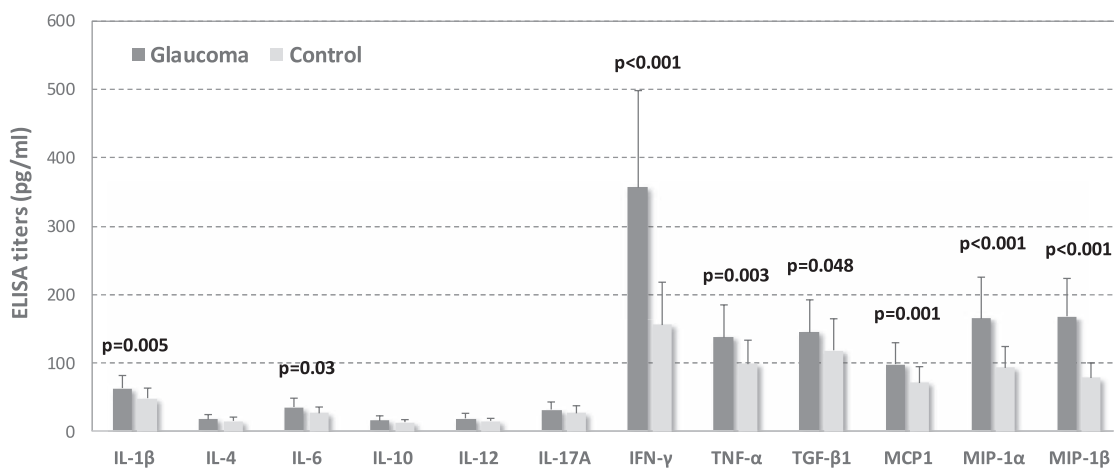


**FIGURE 5.** In vitro analysis of T lymphocytes in glaucoma. In vitro proliferative activity of T cells was also analyzed after stimulation in the presence of retinal antigens from glaucomatous or nonglaucomatous human donors. Flow cytometry-based analysis indicated that the T cells obtained from glaucomatous patients' blood presented a greater rate of proliferation (as assessed by the percentage of CD4+/EdU+ cells) when incubated with glaucomatous human retinal antigens than when incubated with nonglaucomatous retinal antigens. Data are presented as mean ± SD. *P* values were obtained by ANOVA.

suggested that experimental increase in intraocular pressure may also induce T-cell infiltration into the retina, leading to a prolonged phase of retinal ganglion cell degeneration.<sup>21</sup> In addition, transfer of the mononuclear cells obtained from eight glaucoma patients with optic disc hemorrhages into immune-deficient NOD/scid/IL-2R $\gamma$  mice has resulted in a marked decrease of retinal ganglion cell density (Kuehn MH, et al. *IOVS* 2018;59:ARVO E-Abstract 3731). Based on the present findings, it would be interesting to also study whether transfer of Tregs

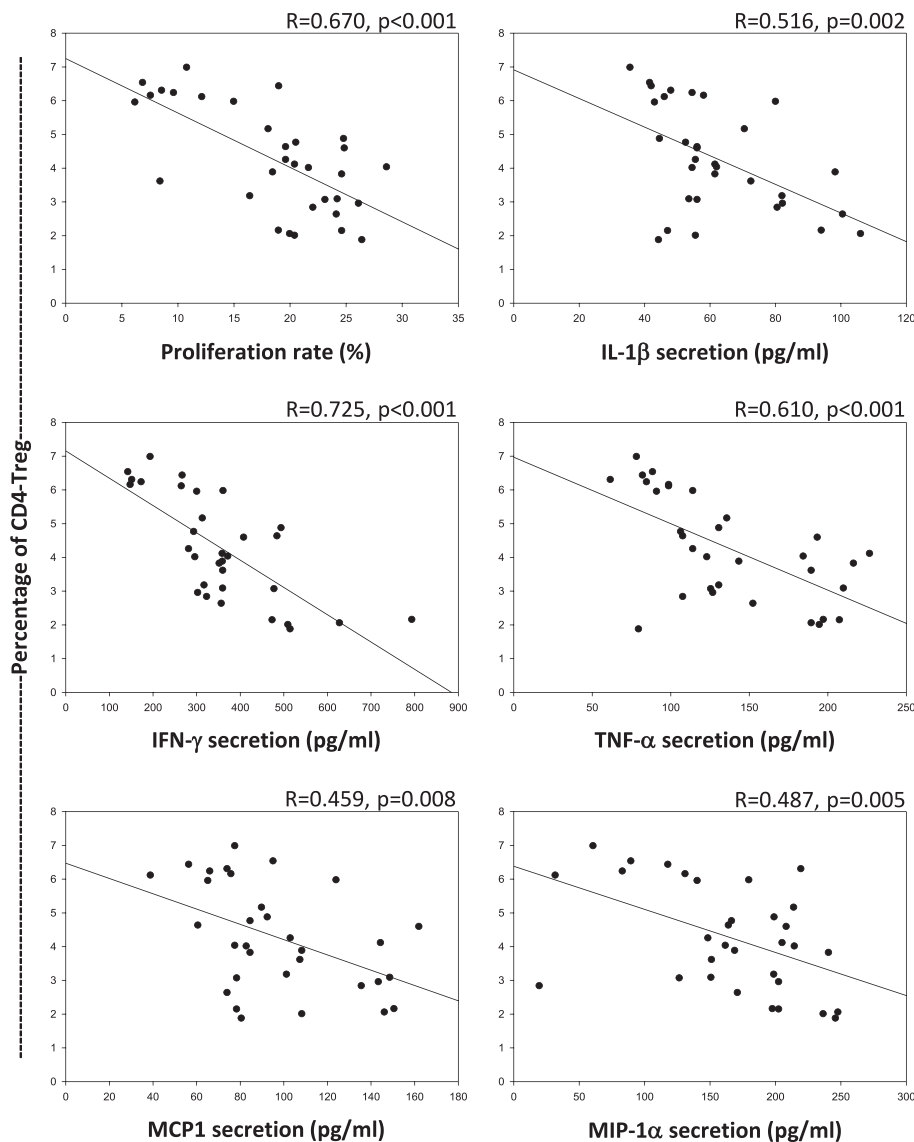
could provide protection against neuroinflammatory and neurodegenerative outcomes in experimental glaucoma. This may open up a new path, through which, similar to other models of neurodegeneration,<sup>70-72</sup> boosting the endogenous Treg response, or adoptive cell therapy by induced Tregs,<sup>76</sup> may be tested as an immunomodulation strategy for glaucoma.

Studies of clinical samples may have some vulnerabilities, inevitably; however, the patient selection strategy in this study aimed to minimize the effects of individual heterogeneity. The



**FIGURE 6.** In vitro analysis of T lymphocytes in glaucoma. T-cell activity was also determined by analysis of cytokine/chemokine secretion in culture supernatant. Cytokine/chemokine secretion of T cells from glaucomatous blood ( $n = 32$ ) was greater than from controls ( $n = 21$ ) as determined by a multianalyte ELISArray. Compared to control samples, molecules exhibiting a significantly increased secretion in glaucomatous samples included IL-1 $\beta$ , IL-6, IFN- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$ 1, MCP1/CCL-2, MIP-1 $\alpha$ /CCL-3, and MIP-1 $\beta$ /CCL-4. Data are presented as mean ± SD, and *P* values are shown when there is statistically significant difference by ANOVA.





**FIGURE 7.** In vitro analysis of T lymphocytes in glaucoma. Presented are some of the scatterplots showing a significant relationship between the ratio of CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> Tregs (CD4<sup>+</sup>Tregs) in glaucomatous samples ( $n = 32$ ) and the rate of in vitro proliferation (as assessed by the percentage of CD4<sup>+</sup>/EdU<sup>+</sup> cells), or ELISA titers of secreted proinflammatory cytokines or chemokines in culture. Data were obtained by linear regression analysis.

study groups were matched and distribution balanced for demographic variables; and systemic or ocular diseases, which could influence the analyses, were excluded. However, glaucoma patients were typically using a combination of eye drops to lower their elevated intraocular pressure. Although we did not detect any relationship between T-cell subset distribution and the number or type of intraocular pressure-lowering treatment in studied patients, a typically used prostaglandin analog, compared to a  $\beta$ -adrenergic blocker, has previously been found to reduce aqueous humor concentration of TGF- $\beta$ 1 in patients with exfoliative glaucoma.<sup>78</sup> In contrast, there is evidence of increased TGF- $\beta$ 1 in blood samples of patients with primary open-angle glaucoma.<sup>46,49</sup> Such a glaucoma-related increase in TGF- $\beta$ 1 (an anti-inflammatory cytokine predominantly secreted by Tregs) would actually be expected to induce Tregs,<sup>60</sup> but the present study detected their lower frequency in glaucomatous samples rather than in controls. Clarification of this seemingly conflicting aspect

needs further studies. Another limitation of the present study was related to its cross-sectional design. With respect to variabilities between individual patients with glaucoma and the asynchronicity of neuron loss over a chronic disease period, longitudinal studies are expected to provide more useful information about the relationship of T-cell responses to glaucoma progression. The presented findings encourage the continued study of the glaucoma group, longitudinally, to thereby further expand the present information.

In conclusion, the decreased frequency of CD4<sup>+</sup> Tregs among glaucoma patients, along with the increased inflammatory stimulation of CD4<sup>+</sup> T lymphocytes, may suggest a shift in T-cell homeostasis. The findings presented stimulate further studies of immune responses in different directions including pathogenic mechanisms, immunotherapies, and diagnostics in glaucoma. First, longitudinal information from analysis of larger patient cohorts with glaucoma should help determine whether dysregulated T-cell responses are associated with disease

progression. Second, by supporting the clinical relevance of recent experimental data, the presented findings motivate continued studies of experimental models to further explore the complex interplay of glaucomatous neurodegeneration with immune regulation, so that new treatment modalities may be developed for immunomodulation. Third, future studies should also determine whether the T-cell subset imbalance detectable in blood samples may serve as a “biomarker of autoimmune susceptibility for glaucoma.” Owing to the etiologic complexity of glaucoma and the significant variability among patients, rather than a single biomarker, a set of biomarkers may collectively provide improved information for clinical prediction of glaucoma or follow-up of treatment responses. Analysis of T lymphocytes in blood samples may therefore complement the information from analysis of previously suggested biomarker candidates (specific molecules,<sup>41,79,80</sup> oxidative stress-related candidates,<sup>81</sup> and perhaps more). To determine whether T-cell subset profiling may be useful for clinical testing of glaucoma, longitudinal studies of larger and heterogenous patient groups are required.

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