

Polypoidal Choroidal Vasculopathy Associate With Diminished Regulatory T Cells That Are Polarized Into a T Helper 2-Like Phenotype

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PURPOSE. To investigate possible roles of T helper (Th) cells, regulatory T cells (Tregs), and the recently mapped Th-like Tregs in patients with polypoidal choroidal vasculopathy (PCV).

METHODS. In this prospective case-control study, we obtained fresh venous blood from patients with PCV ($n = 24$), age-matched healthy controls ($n = 32$), and patients with neovascular AMD ($n = 45$). All participants underwent a comprehensive ocular examination including fluorescein and indocyanine green angiography for where retinal disease was suspected. Using flow cytometry, we identified Th subsets, Tregs, and Th-like Tregs. Plasma samples were stored at -80°C to investigate plasma cytokines of interest.

RESULTS. Compared to healthy controls, patients with PCV had lower percentages of Tregs ($8.7\% \pm 2.8\%$ vs. $7.3\% \pm 1.7\%$, $P = 0.027$), which were significantly more Th2-like polarized ($42.6\% \pm 13.3\%$ vs. $50.5\% \pm 13.0\%$, $P = 0.029$). These changes differed from that observed in neovascular AMD, which compared to healthy controls had fewer Th1/Th17 cells ($3.6\% \pm 2.7\%$ vs. $2.4\% \pm 2.5\%$, $P = 0.049$), comparable Treg levels, and no distinct polarization of Th-like Tregs. Because of these findings, we measured plasma IL-4 and IL-33 levels. Plasma IL-33 in patients with PCV (median 0.30 pg/mL) was twice as high compared to healthy controls (median 0.16 pg/mL; $P = 0.037$).

CONCLUSIONS. PCV associate with diminished Tregs that are polarized more into a Th2-like phenotype. This is correlated to IL-33 levels, which we also find increased in patients with PCV. Our findings suggest a possible role for Th2-like Tregs and IL-33 in PCV.

Keywords: polypoidal choroidal vasculopathy, neovascular age-related macular degeneration, t cells, interleukin-33

Polypoidal choroidal vasculopathy (PCV) is an exudative maculopathy first described in 1982 by Lawrence Yannuzzi.¹ The main features of PCV are the branching choroidal vessels and the aneurysm-like acquired polypoidal lesions.^{2,3} Massive hemorrhages are common and can originate from polypoidal ruptures.^{4,5} In patients with an initial diagnosis of neovascular AMD (nAMD) who undergo indocyanine green angiography (ICGA), PCV is seen in 5% to 10% of Caucasians and in ~50% of Asians.^{4,6,7} Together, PCV and neovascular AMD are the most common reason of irreversible vision loss in developed countries.^{4,7}

Aging, drusen-formation, complement, and immune dysfunction facilitate development of AMD.⁸⁻¹¹ Progression to late-stage AMD with choroidal neovascularization (CNV) is the consequence of a complex immunological play: ocular aging, immunologic aging, and immunologic dysfunction promote angiogenesis through VEGF expression.⁸⁻¹¹ Etiologic aspects of PCV remains largely unknown. Tong et al.¹² found increased intraocular VEGF levels in both neovascular AMD and PCV, but significantly lower in PCV than in neovascular AMD. Zeng et

al.¹³ found higher levels of serum matrix metalloproteinases 2 and 9 in patients with PCV than in patients with neovascular AMD. Lafaut et al.¹⁴ identified islands of lymphocytic infiltration around the aneurysmal vessel dilations in PCV specimens. Although these findings give some insight and suggest a possible role for immunologic dysfunction in PCV, the larger picture remains unclear.

Studies of experimental CNVs on mice and observational studies in patients with neovascular AMD suggest that blood leukocytes are important for CNV formation.¹⁵⁻²³ Lechner et al.²⁰ found that complement proteins C3a, C4a, and C5a were significantly increased in plasma of patients with neovascular AMD, but not in patients with PCV. Liu et al.²¹ found that complement protein C5a promotes CD4⁺ T cell expression of IL-17 and IL-22. In addition, in vitro studies demonstrate that activated T cells upregulate RPE expression of complement proteins and chemokines.^{22,23} These interesting findings propel an attention toward T cells in AMD research.

T helper (Th) cells are CD4⁺ T cells that release cytokines to orchestrate immunologic activity of other immune cells.²⁴ Th



cells can be categorized into functional subtypes: Th1 cells promote activities of macrophages and cytotoxic T cells, Th2 cells promote activities of B cells and eosinophils, and Th17 cells produce IL-17 and promote inflammation and autoimmunity. Regulatory T (Treg) cells are CD4⁺ T cells that modulate the immune system, including the activities of the Th cells.²⁵ In 2012, Duhon et al.²⁶ described subpopulations of Tregs mimicking Th cells and Halim et al.²⁷ recently characterized these Th-like Treg subsets in functional details. Neither Th subtypes, Tregs, or Th-like Treg subtypes have been characterized in patients with PCV.

Our aim with this study was to map these aspects through blood analysis of patients with PCV and compare to healthy controls and patients with neovascular AMD. Interestingly, patients with PCV had lower percentage of Tregs, which in turn were more Th2-like. Because of these findings, we decided to quantify plasma levels of the cytokines IL-4 and IL-33: IL-4 induces Th polarization toward Th2 in general,²⁴ whereas IL-33 has been implicated in a range of immune-mediated as it causes the differentiation of Treg cells into Th2-like Treg cells.²⁸ Patients with PCV did not differ in plasma IL-4, but had twice as high plasma IL-33. Taken together, we here present important immunologic insight into a poorly understood retinal disease.

METHODS

Study Design and Ethics

All aspects of this prospective case-control study follow the tenets of the Declaration of Helsinki and ethical approval was obtained by the Regional Committee of Ethics in Research of the Region of Zealand (SJ-379). Prior to participation, participants had the nature of the study explained and gave oral and written informed consent. Participants were recruited consecutively from our outpatient clinic at Department of Ophthalmology, Zealand University Hospital, Denmark. Based on our previous study on CD4⁺CXCR3⁺CCR6⁺ T cells in patients with neovascular AMD,¹⁷ we calculated that at least 18 participants in each group was necessary to obtain sufficient power (assuming $\alpha = 0.05$, $\beta = 0.8$, and $\sigma = 2$). Therefore, we recruited at least 18 participants in each group and stopped further recruitment after a total of 110 participants.

Participant Eligibility, Clinical Information, and Retinal Diagnosis

Participants were recruited if they had either PCV in one or both eyes, neovascular AMD in one or both eyes, or healthy retinas in both eyes (healthy controls). Healthy controls were recruited among biologically unrelated relatives to the patients. This was an intentional strategy to better match the control group to the patient groups, as we have previously found that lifestyle factors can potentially influence systemic immunology.^{29,30} Participants were included if they fulfilled following criteria to avoid ongoing immune activity: no cancer, no immune disease, no infectious disease, and no chemo- or immunotherapy for any reason. Because this study investigated Th cell polarization, we also did not include any participants with asthma. Patients with recent onset of CNV were not included because of acute immune activity.¹⁹ Patients in ranibizumab or aflibercept therapy were only included 4 or 8 weeks after since last injection, respectively, to avoid potential interaction with the antibodies used for flow cytometry. All participants were interviewed to obtain data on lifestyle and medical history. Lifestyle factors noted were smoking habits, alcohol consumption, body mass index, and physical activity.³⁰

Medical data were crosschecked with the electronic patient record to ensure accuracy. Participants were examined using slit-lamp biomicroscopy, digital fundus photography, and spectral-domain optical coherence tomography (OCT), and retinal angiography (both fluorescein and indocyanine green) where CNV was suspected. We used the following definition for our groups:

- Healthy controls: Less than 10 small drusen (diameter <63 μm) and no pigment abnormalities.³¹
- Neovascular AMD: Fibrovascular pigment epithelium detachments and choroidal neovascular membranes with subretinal or sub-RPE hemorrhages or fibrosis.³¹
- PCV: One or more polyps in early-phase ICGA with a hypofluorescent halo and with/without BVN. Other stigmata used to support the diagnosis were orange-red focal subretinal polyp-like structures, pulsation of polyps on ICGA video, and a protrusion from the choroid elevating RPE from the Bruch's membrane observed on OCT.⁷

Blood Sampling

Venous blood was sampled from antecubital veins in separate EDTA and lithium-heparin coated tubes. EDTA stabilized blood was used for flow cytometric analyses within 4 hours after blood sampling. One lithium-heparin coated tube was used for routine C-reactive protein measurement. Other tubes with lithium heparin stabilized blood were centrifuged for 15 minutes at 1500 G after which plasma was isolated and stored at -80°C for later quantification of plasma cytokines.

Flow Cytometry

We obtained the white blood cell count using an automated hematology analyzer (Sysmex KX-21N; Sysmex Corp., Kobe, Japan) to calculate blood volume necessary to obtain 5×10^5 white blood cells in each test tube. The red blood cells were lysed in 1% lysis buffer (Nordic Biosite AB, Täby, Sweden) for 10 minutes in the dark at room temperature. We then washed the cells three times by first centrifuging for 5 minutes at 500 g, decanting the supernatant, and then resuspending the cells in an isotonic buffer (BD FACSFlow; BD Biosciences, Franklin Lakes, NJ, USA). We then added marker-specific monoclonal antibodies to the sample tube and added fluorochrome-matched negative isotypes were added to a separately prepared tube (Supplementary File S1). Tubes were incubated in dark at room temperature for 20 minutes, after which the cells were washed and resuspended in an isotonic buffer (BD Biosciences). Stained cells were analyzed using flow cytometry (BD Biosciences) with a sample size gated for 100,000 singlet leukocytes. We used analytical software (Kaluza version 1.5.20365.16139; Beckman Coulter Inc., Pasadena, CA, USA) for all flow cytometric analyses. Two independent evaluators (YS, MKN) performed all analyses completely blinded to each participant's condition and each other. CD4⁺ T cells were identified and gated based on their CXCR3 and CCR6 expression (Supplementary File S2). Zhang et al.³² studied Th cell subsets in humans and found that CD4⁺CXCR3⁺CCR6⁻ cells had characteristics of Th1, CD4⁺CXCR3⁻CCR6⁻ had characteristics of Th2, and that CD4⁺CXCR3⁻CCR6⁺ had characteristics of Th17. We also measured the CD4⁺CXCR3⁺CCR6⁺ cells, which may reflect the Th1/Th17 cell subset (Supplementary file S2).²⁷ Treg cells were identified as CD4⁺CD127^{low}CD25^{high} cells (Fig. 1),¹⁸ and Th subsets in Treg cells (Th-like Tregs) were determined (Supplementary File S3).²⁷ Nonspecific signaling was elimi-

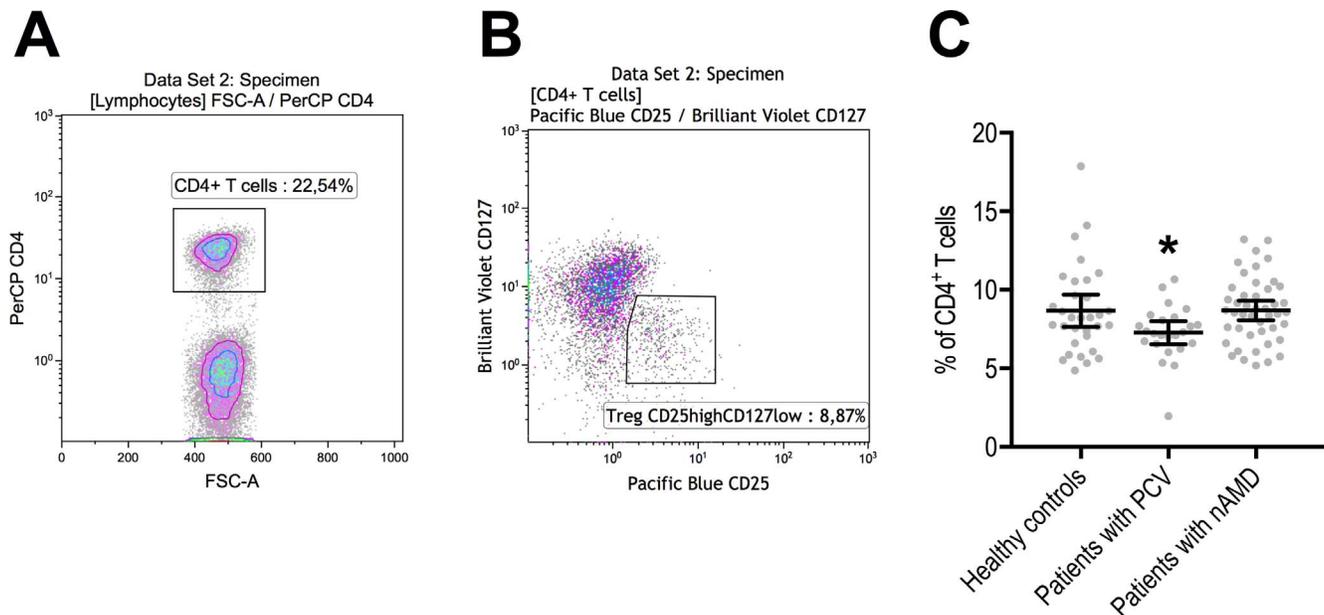


FIGURE 1. Identification and quantification of Treg and comparison between healthy controls and patients with PCV or patients with nAMD. (A) CD4⁺ T cells were identified as CD4⁺ lymphocytes. (B) Treg were identified as CD4⁺ T cells that were CD25^{high} and CD127^{low}. (C) Treg levels did not differ significantly between healthy controls and patients with nAMD ($P = 0.96$, independent samples t -test), but was significantly lower in patients with PCV than in healthy controls ($P = 0.027$, independent samples t -test, signified with an *asterisk*). Whiskers represent mean and 95% confidence interval.

nated using the corresponding negative isotype control at a threshold of 1%.

Plasma Cytokine Assays

IL-4 and IL-33 were quantified using the commercially available human assays from MSD (U-PLEX, Meso Scale Diagnostics, Rockville, MD, USA). The assays were performed per the manufacturer's instructions and recommendations (Supplementary File S4). Prepared plates were immediately read on a commercial instrument (QuickPlex SQ120; Meso Scale Diagnostics), which converts luminescence values to plasma concentrations of the cytokines measured based on the predefined concentrations in calibrators. Duplicate measurements allowed sample-specific calculation of the coefficient of variation (CV), which we used as a guide to reanalyze any samples with high CV (>20%). Overall quality was satisfactory (CV mean \pm SD: IL-4 = 5.6% \pm 4.4%; IL-33 = 5.6% \pm 4.5%). No extreme outliers were found for plasma IL-4. Two cases of extreme outliers (2.3 pg/mL and 8.7 pg/mL) for plasma IL-33 were excluded from the analyses.

Statistical Analysis

All statistical analyses were made using statistical software (SPSS version 23; IBM Corp., Armonk, NY, USA). Mean and standard deviation (SD) and parametric tests were used where normal distribution was present, and otherwise median and interquartile range (IQR) and nonparametric tests were used. Categorical variables were presented in numbers and percentages and compared between groups using the χ^2 test or Fisher's exact test when dealing with small categories ($n < 5$). P -values below 0.05 were interpreted as sign of statistical significance. Figures were made using graphing software (Prism version 7; GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Study Population

We recruited 110 participants in total. Nine were excluded due to suspected ongoing immune response or because samples failed flow cytometry (Supplementary File S5). Remaining participants were all included for our analyses (Table 1). Groups did not differ significantly in demographics, comorbidities, and lifestyle factors (Table 1).

Differences in CD4⁺ T Helper Cell Polarization

Compared to healthy controls, fewer Th1/Th17 cells (CXCR3⁺CCR6⁺) were observed in patients with neovascular AMD ($P = 0.049$, independent samples t -test; Table 2). Patients with PCV did not differ significantly from healthy controls, but we did observe a trend toward higher Th2 ($P = 0.059$, independent samples t -test).

Lower Tregs and Higher Th2-like Tregs in Patients With PCV

We used CD25^{high} and CD127^{low} in CD4⁺ lymphocytes to identify and quantify the Treg population (Fig. 1). In healthy controls, we found that mean (SD): 8.7% (2.8%) of CD4⁺ T cells were Tregs, which was similar to the level in patients with neovascular AMD (mean: 8.7%, SD: 2.1%, $P = 0.96$, independent samples t -test). Patients with PCV had significantly fewer Tregs than healthy controls (mean: 7.3%, SD: 1.7%, $P = 0.027$, independent samples t -test) with a significantly lower distribution variance ($F = 4.029$, $P = 0.050$, Levene's test for equality of variances).

In Tregs, we investigated Th-like populations (Supplementary File S3). Healthy controls and patients with neovascular AMD did not differ significantly in any of the measured

TABLE 1. Participant Characteristics

	Healthy Controls (n = 32)	Patients with PCV (n = 24)	Patients With nAMD (n = 45)	P Value
Demographics				
Age (y), mean (SD)	73.4 (7.9)	72.3 (7.7)	75.4 (7.2)	0.24*
Females, n (%)	18 (56)	16 (67)	24 (53)	0.56†
Comorbidities				
Hypertension, n (%)	11 (34)	11 (46)	24 (53)	0.26†
Cardiovascular disease, n (%)	9 (20)	5 (21)	9 (16)	0.85†
Hypercholesterolemia, n (%)	12 (38)	9 (38)	11 (24)	0.37†
Type 2 diabetes, n (%)	0 (0)	3 (13)	6 (13)	0.057‡
Lifestyle factors				
Smoking, n (%)				0.29‡
Active	5 (15)	8 (33)	13 (29)	
Previous	14 (44)	12 (50)	18 (40)	
Never	13 (41)	4 (17)	14 (31)	
Alcohol consumption, units, median (IQR)	4 (2-7)	4 (1-14)	4 (1-9)	0.72§
Body mass index, mean (SD)	25.3 (4.4)	25.3 (3.7)	26.1 (4.2)	0.73*
Physically active, n (%)	22 (69)	13 (54)	24 (53)	0.36†

Smoking habits were categorized into one of three groups: active, previous (>100 cigarettes during lifetime and ceased smoking >12 months), or never. Alcohol consumption is quantified using units/week (1 unit = 15 mL/12 g pure ethanol). Body mass index is calculated as weight divided by height squared. Being regularly physically active is categorized using a simple question for epidemiologic studies previously validated in Danish patients with AMD.²⁹

* Statistical comparisons are made using 1-way ANOVA.

† Statistical comparisons are made using χ^2 -test.

‡ Statistical comparisons are made using Fisher's Exact test because of categories with ≤ 4 cases.

§ Statistical comparisons are made using Kruskal-Wallis' test.

populations (Table 3). Patients with PCV had significantly higher Th2-like Treg ($P = 0.029$, independent samples t -test) and significantly lower Th17-like Treg ($P = 0.039$, independent samples t -test).

Higher Plasma IL-33 in Patients With PCV

Because of our findings on Th2-like Treg in patients with PCV, we shifted our focus to plasma levels of cytokines IL-4 and IL-33. Plasma IL-4 did not differ between healthy controls and patients with PCV or neovascular AMD (Fig. 2). Patients with PCV had significantly higher and almost twice as high median IL-33 0.30 pg/mL compared to the healthy controls (median 0.16 pg/mL; $P = 0.037$, Mann-Whitney U test), while patients with neovascular AMD had levels comparable to that in healthy controls ($P = 0.55$, Mann-Whitney U test; Fig. 2). In patients with PCV, where we observed a higher IL-33 and Th2-like Treg cells, we also found a correlation trend between higher plasma IL-33 and higher percentage of Th2-like cells in the Treg population ($\rho = 0.4$, $P = 0.062$, Spearman's correlation).

Patient Characteristics in Relation to Immunologic Findings

Since we observed a trend ($P = 0.057$, Fisher's Exact test) toward higher prevalence of type 2 diabetes as a comorbidity in patients with PCV and patients with neovascular AMD, we compared all our findings between those with and without type 2 diabetes and did not find any significant differences ($P > 0.1$ for all comparisons).

Expectedly, groups differed significantly in the proportion of AREDS2-based supplement users ($n = 3$ [13% of patients with PCV]; $n = 13$ [29% of patients with neovascular AMD]; and $n = 0$ [0% of healthy controls], $P < 0.001$, Fisher's Exact test). To investigate if AREDS2-based supplement use influenced our immunological measurements, we compared patients who used AREDS2-based supplements to patients who did not use AREDS2-based supplements (Supplementary File S6). No differences were observed in patients with PCV. In patients with neovascular AMD, plasma IL-4 was significantly higher among AREDS2-based supplement users (median 0.30 pg/mL; IQR: 0.11-0.38 pg/mL) than those who did not use

TABLE 2. Comparison Between Healthy Controls and Patients With PCV or Patients With nAMD in CD4⁺ T Cell Expression of T Helper Cell Markers CXCR3 and CCR6

	Healthy Controls	Patients With PCV	P Value*	Patients With nAMD	P Value†
CD4 ⁺ CXCR3 ⁺ CCR6 ⁻ (Th1)	4.2 (3.6)	4.5 (3.4)	0.76	3.1 (2.2)	0.11
CD4 ⁺ CXCR3 ⁻ CCR6 ⁻ (Th2)	63.6 (10.9)	69.4 (11.6)	0.059	68.8 (11.8)	0.053
CD4 ⁺ CXCR3 ⁻ CCR6 ⁺ (Th17)	28.6 (9.4)	23.3 (10.5)	0.52	25.8 (10.2)	0.22
CD4 ⁺ CXCR3 ⁺ CCR6 ⁺ (Th1/Th17)	3.6 (2.7)	2.7 (2.7)	0.26	2.4 (2.5)	0.049

All values are presented as a percentage of the total CD4⁺ T cell population in means and standard deviations in parentheses. Boldface P values indicate statistically significant differences from healthy controls.

* Statistical comparisons are made using independent samples t -test between healthy controls and patients with PCV.

† Statistical comparisons are made using independent samples t -test between healthy controls and patients with neovascular AMD.

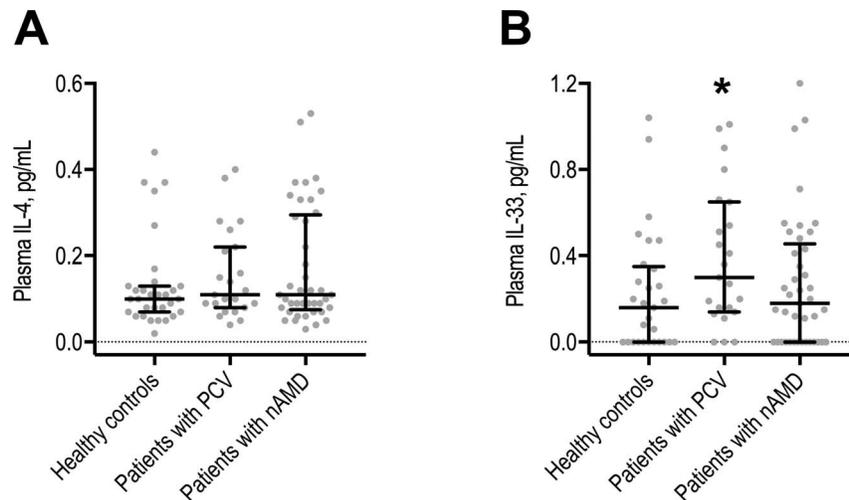


FIGURE 2. Comparison between healthy controls and patients with PCV or patients with nAMD in plasma levels of IL-4 and IL-33. **(A)** Plasma IL-4 levels did not differ significantly between the groups. *Whiskers* represent median and interquartile range. **(B)** Plasma IL-33 levels did not differ significantly between healthy controls and patients with nAMD ($P = 0.55$, Mann-Whitney U test), but was significantly higher in patients with PCV than in healthy controls ($P = 0.033$, Mann-Whitney U test, signified with an *asterisk*). *Whiskers* represent median and interquartile range.

AREDS2-based supplements (median 0.09 pg/mL; IQR: 0.07–0.13 pg/mL; $P = 0.002$, Mann-Whitney U test).

Patients were followed in the clinic in median 16 months (IQR: 6–34 months) prior to blood sampling (patients with PCV: median 16 months [IQR: 4–34 months]; patients with neovascular AMD: median 16 months [IQR: 9–32 months]; $P = 0.313$, Mann-Whitney U test). We found no correlations between time from disease presentation to sampling and to any of our immunologic measurements ($P > 0.1$ for all correlations, Spearman's correlation). To study disease duration in a different manner, we stratified our patients with PCV according to whether they had polypoidal lesions with strong presence of BVNs (where we assume that the disease is relatively more advanced; $n = 14$) or polypoidal lesions without or very faint BVNs (where we assume that the disease is at an earlier stage; $n = 10$; Supplementary File S7). These groups did not differ significantly in any of the immunologic measurements.

DISCUSSION

In line with our previous findings in patients with neovascular AMD,^{17,33} we find association between neovascular AMD and lower CD4⁺CXCR3⁺ and CD4⁺CXCR3⁺CCR6⁺. CXCR3⁺ T cells are particularly interesting since they migrate toward areas of inflammation,³⁴ CXCR3-CXCL10 interaction activates downstream pathways that inhibits VEGF-induced endothelial

motility and tube formation,³⁵ and age-related parainflammation in RPE leads to downregulation of CXCL10 expression.³⁶ Dysregulation of CXCR3-CXCL10 axis have been suggested as a biomarker for AMD specifically and a therapeutic target in diseases with uncontrolled angiogenesis in general.^{33,35,37} Dysregulation of CXCR3 in CD4⁺ T cells in patients with neovascular AMD may be specific to the CCR6⁺ subset and reflect a lower systemic Th1/Th17 population. Experimental studies of the Th1/Th17 population in patients with AMD are warranted to understand its contribution for CNV development.

Dysregulation of CXCR3 in CD4⁺ T cells did not seem to play a significant role in PCV. Instead, our results suggest that PCV is a disease characterized by diminished Tregs, increased Th2-like polarization of the Tregs, and increased plasma IL-33.

Balancing an effective immune response with self-tolerance and setting the proper magnitude of the immune response is the key role of Tregs.³⁸ Hence, diminished or dysfunctional Tregs are linked to diseases with an autoimmune component (e.g., allergies and asthma).^{39,40} This regulatory activity is also present in the retina, where RPE induces Tregs to suppress the intraocular activity of proinflammatory leukocytes.⁴¹ RPE induced Tregs secrete high levels of immunoregulatory cytokines which suppress Th1 and Th17 activity.⁴² These mechanisms shed explanatory light on our results: well-functioning Treg activity in patients with neovascular AMD may lead to Th1/Th17 downregulation to dampen the ongoing

TABLE 3. Comparison Between Healthy Controls and Patients With PCV or Patients With nAMD in Treg Th-like Cells

	Healthy Controls	Patients With PCV	P Value*	Patients With nAMD	P Value†
CD4 ⁺ CD25 ^{high} CD127 ^{low} CXCR3 ⁺ CCR6 ⁻ (Th1-like Treg)	2.5 (2.2)	2.3 (1.8)	0.69	1.8 (1.6)	0.12
CD4 ⁺ CD25 ^{high} CD127 ^{low} CXCR3 ⁻ CCR6 ⁻ (Th2-like Treg)	42.6 (13.3)	50.5 (13.0)	0.029	45.4 (14.9)	0.39
CD4 ⁺ CD25 ^{high} CD127 ^{low} CXCR3 ⁻ CCR6 ⁺ (Th17-like Treg)	51.4 (12.6)	44.6 (11.7)	0.039	50.4 (14.4)	0.72
CD4 ⁺ CD25 ^{high} CD127 ^{low} CXCR3 ⁺ CCR6 ⁺ (Th1/Th17-like Treg)	3.4 (2.9)	2.6 (2.6)	0.29	2.4 (2.1)	0.069

All values are presented as percentage of the total Treg population in means and standard deviations in parentheses. Bold P values indicate statistically significant differences from healthy controls.

* Statistical comparisons are made using independent samples t -test between healthy controls and patients with PCV.

† Statistical comparisons are made using independent samples t -test between healthy controls and patients with neovascular AMD.

proinflammatory angiogenic activity. Diminished Tregs in patients with PCV allow a different direction of angiogenesis and may explain why studies find series of intraocular cytokine expression in PCV.^{43,44} Zhao et al.⁴³ found significantly higher levels of the proinflammatory IL-1 β in the vitreous of eyes with PCV when compared to controls (eyes with idiopathic epiretinal membrane) and patients with neovascular AMD. Sasaki et al.⁴⁴ found significantly higher levels of a range of proinflammatory cytokines in the aqueous of eyes with PCV, including IL-4 which suggests a local Th2-like response. Is PCV a Th2-mediated disease? Gold-standard diagnosis of PCV requires ICGA and consequently very few epidemiologic studies exist; therefore, it is currently not possible to draw clear links to other Th2- or Th2-like Treg-related diseases.

In 2015, MacDonald et al.²⁸ reported the first Th2-like Treg-associated disease in humans (systemic sclerosis: an autoimmune disease with dysfunctional angiogenesis despite increased VEGF).²⁸ Levels of Tregs were similar in patients with systemic sclerosis when compared to healthy controls, but the Tregs in patients were more CXCR3⁻CCR6⁻ and expressed Th2-associated cytokines. Halim et al.²⁷ found that Th2-like Tregs have greater migratory ability and a higher viability and blasting capacity through STAT5 phosphorylation, which promotes angiogenesis.⁴⁵ Treg polarization into Th2-like phenotype is facilitated by IL-33.^{28,46} Although no previously published reports have investigated IL-33 in patients with PCV, it is interesting to note that Genentech, Inc. have applied for and received a patent for the use of anti-IL-33 for PCV.⁴⁷

Pachychoroid neovascularopathy is a new clinical definition of CNV-diseases associated with a thick dilated choroid, wherein PCV constitutes a large and important proportion of cases.⁴⁸ By post-hoc reviewing our PCV cases using the definition by Miyake et al.,⁴⁹ 17 (71%) patients with PCV could be classified with the diagnosis pachychoroid neovascularopathy. We found that these patients do not have AMD-like features of lower CXCR3⁺ and CXCR3⁺CCR6⁺, but have lower Tregs than healthy controls ($P = 0.014$), which were increasingly Th2-like polarized ($P = 0.036$), and had increased plasma IL-33 levels (median 0.45, IQR: 0.16–0.66) at a near-significant level ($P = 0.060$). We here provide rare immunologic insight into pachychoroid neovascularopathy and together with the genetic study by Miyake et al.,⁴⁹ our collective results suggest that pachychoroid neovascularopathy may be etiologically distinct from neovascular AMD.

Important limitations of this study should be considered. First, the observational, cross-sectional, and exploratory nature of this study can only correlate possible mechanisms and we cannot infer conclusively on causality. Second, Th subtypes are distinguished by their functional properties (e.g., cytokine secretion). We did not perform functional analyses of Th subtypes defined in this study, but instead relied on the accuracy of the functional studies made by others.^{27,28} Third, we did not find any relationship between disease duration and the immunological findings, but these calculations are based on the assumption that time of disease onset equals the time of disease presentation at our clinic. Theoretically, we cannot know how long the disease was present prior to presentation at our clinic. Quiescent lesions—especially small peripheral polyps without additional features such as hemorrhages—may be relatively asymptomatic, at least in the beginning stages of the disease. Finally, investigations of systemic immunity in elderly patients must carefully select their control group since lifestyle factors may significantly influence the results.³⁰ A strength of this study was our strategy of recruiting among biologically unrelated relatives to the patients to better match the control group in terms of lifestyle factors. We measured several aspects of lifestyle factors to provide insight into the validity of this approach (smoking, body mass index, alcohol

use, and physical activity), which all were comparable between the groups. However, considering that dietary habits also may play a role in AMD,^{50–52} further validation of the strength of this control group recruitment strategy should include an assessment of dietary habits.

Taken together, we conclude that PCV associates with systemic immunologic changes that differ from that seen in neovascular AMD. Patients with PCV have diminished Tregs that are increasingly polarized into a Th2-like phenotype wherein IL-33 secretion may play an important role. Experimental studies are warranted to determine causality and whether these immunologic alterations can be used as targets for treatment.

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