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Peripheral blood CD163(+) monocytes and soluble CD163 in dry and neovascular age-related macular degeneration

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Abstract

Macrophages are the main infiltrating immune cells in choroidal neovascularization (CNV), a hallmark of the human wet, or neovascular age-related macular degeneration (AMD). Due to their plasticity and ability to adapt to the local microenvironment in a tissue-dependent manner, macrophages display polar functional phenotypes characterized by their cell surface markers and their cytokine profiles. We found accumulation of hemoglobin-scavenging cluster of differentiation 163 (CD163)(+) macrophages in laser-induced CNV lesions and higher expression of CD163(+) monocytes in the peripheral blood on day 7 post injury in mice. In comparison, CD80(+) macrophages did not differ with laser-injury in young or aged mice and did not significantly change in the peripheral blood of CNV mice. We examined the percentages of CD163(+), CD206(+), and CD80(+) monocytes in the peripheral blood of patients with wet AMD, patients with dry AMD, and in age-matched individuals without AMD as controls. Percentages of peripheral blood CD163(+) monocytes in both dry AMD ($P < .001$) and wet AMD ($P < .05$) were higher than in age-matched non-AMD controls, while there was no difference between the groups in the percentages of peripheral CD206(+) and CD80(+) monocytes. Further, serum level of soluble CD163 (sCD163) was elevated only in patients with wet AMD ($P < .05$). An examination of 40 cytokine levels across the study groups revealed that anti-VEGF treated patients with wet AMD, who showed no exudative signs on the day of blood drawing had a cytokine profile that was similar to that of non-AMD individuals. These results indicate that CD163 could be further evaluated for its potential as a useful marker of disease activity in patients with neovascular AMD. Future studies

Abbreviations: AMD, age-related macular degeneration; BCVA, best-corrected visual acuity; CD163, cluster of differentiation 163; CNV, choroidal neovascularization; IHC, immunohistochemistry; LCNV, laser-induced CNV; OCT, optical coherence tomography; PBS, phosphate-buffered saline; RPE, retinal pigment epithelium.

Narsis Daftarian and Souska Zandi contributed equally to this work.

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will address the origin and potential mechanistic role of CD163(+) macrophages in wet AMD pathologies of angiogenesis and leakage of blood components.

KEYWORDS
AMD pathogenesis, CD206, choroidal neovascularization (CNV), M1/M2 differentiation, macrophage polarization

1 | INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of vision loss in the elderly in the developed countries. Next to age, as the strongest risk factor, the contribution of genes is widely accepted.[1-4] Single nucleotide polymorphisms (SNPs) in the complement factor H (CFH) and PLEKHA1/ARMS2/HTRA1 genes[5,6] account for over 70% of the population at risk.[6] However, currently reliable non-genomic markers for AMD do not exist.

AMD has two types, the dry form that ultimately leads to macular geographic atrophy, and the more rapidly progressing wet form, characterized by choroidal neovascularization (CNV) and leakage. Most AMD cases are of the dry type, which can become exudative upon developing CNV. Both dry and wet AMD ultimately lead to a loss of vision, while wet AMD progresses more rapidly.

Early pathogenesis of AMD involves deposits of material, clinically referred to as drusen under the retinal pigment epithelium (RPE) and on the Bruch's membrane in consequence of an exceeded phagocytotic capacity of the RPE.[7,8] Drusen can grow, merge over time, and erode the RPE and the Bruch's membrane, or disappear without causing retinal atrophy.

AMD pathology has inflammatory and immunologic components,[9-13] in which macrophages play a key role. Macrophages polarize to functional phenotypes, M1, or M2, which depending on the context lead to opposing outcomes of inflammatory reactions.[14,15] Both M1- and M2-type macrophages are present in AMD,[16-18] however, their roles in the pathogenesis are unclear.

We reported an innate-immune shift toward M2-macrophages, and a role for these cells in experimental CNV. Intravitreally injected M2-type macrophages increased, while M1-type macrophages decreased the size of CNV lesions.[18] Moreover, we found significant levels of cluster of differentiation 163 (CD163) in experimental CNV, while unlasered young and aged mice expressed very low levels of CD163.[18] In human donor eyes more CD163(+) macrophages were found in the outer retina, subretinal, and sub-RPE space in patients with AMD, when compared to tissues from healthy controls.[19]

CD163 is a 130 kDa scavenger receptor protein that is proteolytically cleaved and released into the plasma.[20,21] CD163 is cleaved by tumor necrosis factor α-converting enzyme (TACE/ADAM17).[22] Higher levels of soluble CD163 (sCD163) were reported in systemic inflammatory diseases and in diabetes.[23,24] Here, we investigated peripheral CD163(+) monocytes in experimental CNV in mice, and peripheral CD163(+) monocytes and serum level of sCD163 in patients with AMD and in individuals without AMD.

2 | MATERIALS AND METHODS

2.1 | Animals

All animal experiments adhered to The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23) and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male and female C57BL/6J mice (Stock# 000664; Jackson Laboratories, USA or Kyudo Company, Tosu, Saga, Japan), weighing 24-28 g were kept in ventilated cages in a temperature-controlled facility with a 12-hour light/dark cycle and fed standard laboratory chow and water ad libitum. In this study, young WT were 8-12 weeks old, while aged animals were 12 or 16 months old as indicated.

2.2 | Western blot

To obtain tissues, deeply anesthetized animals were perfused with phosphate-buffered saline (PBS) through the heart and eyes were enucleated immediately after perfusion. Choroidal tissue was microsurgically isolated and placed in 100 μL of lysis buffer (mammalian cell lysis kit MCL1, Sigma), supplemented with protease and phosphatase inhibitors (P2850, P5726, P8340 Sigma), and sonicated. The lysate was centrifuged (12 000 rpm, 15 minutes, 4°C) and the supernatant was collected. Each sample was normalized to contain an equal amount of total protein, quantified by protein assay (Bio-Rad

Laboratories), and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene fluoride (PVDF) membranes (Invitrogen). To block nonspecific binding, the membranes were washed with 5% of skim milk and subsequently incubated with antibodies to the following antigens: CD163 (sc-33560, Santa Cruz Biotechnology), CD80 (ab53003, Abcam), and β-tubulin (ab11308, Abcam) at 4°C overnight, followed by incubation with a horseradish peroxidase-conjugated donkey or sheep antibody against rabbit or mouse IgG (NA934V, NXA931, GE Healthcare), or goat anti-rat secondary antibody (goat anti-rat IgG-HRP: sc-2032, Santa Cruz). The signals were visualized by chemiluminescence (ECL kit; GE Healthcare).

2.3 | Laser-induced CNV

A difficulty of studying AMD lies in the lack of a realistic animal model. The laser-induced CNV (LCNV) is a wound healing model that mimics the angiogenesis and leakage aspects of wet AMD. However, it lacks the complex etiology of the human disease.[25] The paucity of satisfactory models has resulted that much research has been based on LCNV. While the insights from LCNV may bear relevance to leakage, angiogenesis, and inflammation, the observations in the LCNV should not directly be extrapolated to the human disease.

To induce CNV, C57BL/6J mice were anesthetized and their pupils were dilated with 5% of phenylephrine and 0.8% of tropicamide. Using a 532-nm laser (Oculight GLx, Iridex), a slit-lamp delivery system, and a cover glass as a contact lens, four spots (100 mW, 50 μm, 100 ms) were placed in each eye. The lesions were located at 3, 6, 9, and 12 o'clock meridians centered on the optic nerve head, and 2-3 disk diameters from the optic nerve head. Development of a bubble under laser radiation confirmed the rupture of the Bruch's membrane. Eyes that showed a hemorrhage in imaging were excluded.

2.4 | Immunohistochemistry

C57BL/6J mice were anesthetized through an, ip, injection of 100 mg/kg ketamine and 10 mg/kg xylazine and perfused with PBS and 4% of paraformaldehyde. The animals' eyes were enucleated, washed with PBS and rapidly frozen in precooled methanol as previously described.[26] After 48 hours the eyes were rehydrated and embedded in optimal cutting temperature compound (Tissue-Tek 4583, Sakura). Sections of 7 μm thickness were prepared using a cryostat (CryoStar NX70, Thermo Scientific). Tissue sections were blocked with 5% of bovine serum albumin (BSA) and 0.3% of Triton X-100 for 90 minutes in room temperature, incubated with 1:200 CD163 monoclonal antibody (ab213612, Abcam) overnight in 4°C, and 1:400 AlexaFluor 488 goat anti-rabbit (A11008, Invitrogen) for 1 hour in room temperature. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, P36971, Invitrogen).

2.5 | Mouse blood flow cytometry

Under anesthesia, peripheral blood was collected from the tail vein of unlasered and lasered mice 7 days after photocoagulation. Erythrocytes were lysed with ammonium-chloride-potassium (ACK) lysing buffer (10-548E; Lonza). Leukocyte cell suspensions were stained with APC anti-mouse CD11b (17-0112-82; eBioscience), FITC anti-mouse CD163 (bs-2527R-FITC; Bioss), and FITC anti-mouse CD80 (11-0801-81; eBioscience). Flow cytometry was performed in a FACS Calibur (BD Biosciences). Data were analyzed using FlowJo software (version 10.1).

2.6 | Human blood flow cytometry analysis

In accordance with the tenets of the "Declaration of Helsinki" an informed and written consent was obtained from all human subjects included in this study. The diagnoses ranged from intermediate to advanced dry AMD and wet AMD, independently established by two board-certified ophthalmologist/retina specialists after complete ocular exams and multimodal imaging. Age- and gender-matched normal controls were selected from patients planned for cataract surgery with no retinal disease after the same complete ocular exams and multimodal imaging. Exclusion criteria for both cases and controls, included systemic and ocular diseases, such as diabetes mellitus, rheumatic disease, active or chronic infectious disease, malignancy, uveitis, retinal vascular occlusive disease, or glaucoma. Complete ocular exams included best-corrected visual acuity (BCVA) test with subjective manifest refraction, complete slit-lamp bio-microscopy examinations, Goldmann applanation tonometry, complete dilated pupil retinal examination using both +90 lens at slit-lamp and indirect ophthalmoscopy by +20 lens. Imaging included optical coherence tomography (OCT) using two different systems (Spectral domain, 3D-OCT 1000MK2, Topcon, Japan, and Spectralis, multimodality diagnostic imaging, Heidelberg, Germany), color fundus photography (Topcon, Japan), fundus autofluorescence, and fluorescein angiography (Spectralis multimodality diagnostic imaging system, Heidelberg, Germany).

Blood was drawn from patients included in the study after informed and written consent. An experienced phlebotomist drew venous blood from case and control subjects referred with inclusion criteria, from August 2017 to August 2018. Blood was collected in EDTA-coated tubes and stored at

4℃ and was experimentally analyzed in flow cytometry <24 hours of being obtained.

Flow cytometry analysis was performed on a Partec CY-flow space instrument (Partec, Germany), equipped with argon laser. A total of 5000 events were acquired in each analysis. FITC-conjugated anti-CD68 (IgG2b-k Cat#562117, Beckton Dickinson, USA) and PE-conjugated anti-CD80 (IgG1-k Cat#557227, Beckton Dickinson), CD163 (IgG1-k Cat#556018, Beckton Dickinson) with the corresponding isotope controls (PE-IgG1-k Cat#555749, FITC-IgG2b-k Cat#555057, Beckton Dickinson) were used. Briefly, 50 μL of peripheral blood with EDTA, as anticoagulant, was used for cell surface receptor labeling. The cells were incubated in dark at room temperature for 20 minutes. Red blood cells were lysed using erythrocyte lysing reagent without fixative (Dako Easylyse, Code S2364). The cells were then washed with PBS and were suspended in 100 μL of PBS and analyzed by flow cytometry and Flomax software (FlowJo, Becton, Dickinson and Company, NJ).

2.7 | Human cytokine and serum analysis

This clinical case series included patients with dry, treated wet AMD, or healthy controls without any relevant systemic or ocular disease (apart from senile cataract), who were scheduled for phacoemulsification surgery and/or vitrectomy. Clinical data regarding ophthalmologic and systemic diagnoses and findings, systemic and local medications were collected. Samples of serum were collected at the beginning of ocular surgery at the Berner Augenklinik am Lindenhofspital, between August 2013 and January 2016. The grading of macular changes was based on clinical findings and OCT diagnostics in dependence on the Clinical Age-Related Maculopathy Staging System (CARMS).[27] The following stages were distinguished: healthy controls (no chorioretinal changes), dry AMD (≥15 intermediate drusen or any large drusen, no intraretinal or subretinal fluid or hemorrhages), and treated wet AMD (signs of exudative AMD, such as subretinal hemorrhage, serous retinal detachments, non-drusenoid RPE detachments, CNV with sub-RPE or subretinal exudations or fibrosis prior to anti-VEGF therapy, or the presence of scars consistent with AMD-treatment).[27] Patients in the treated wet AMD group were subdivided as "dry on the day of blood drawing" and "exudative on the day of blood drawing," meaning that in the former, the CNV lesions had regressed with treatment and in the latter, there was intraretinal and/or subretinal fluid visible in OCT indicating an active CNV, on the day of blood drawing.

Exclusion criteria included a history of systemic malignant, vascular, or inflammatory comorbidities and their treatments, namely, diabetes mellitus or rheumatic diseases; a history of any previous intraocular surgery or ocular trauma in the affected eye or of intraocular inflammation; the presence or history of vitreal/(sub-)retinal hemorrhage; any ocular vascular occlusive disease; or myopia of more than six diopters.

The informed and written consent of all individuals enrolled in this study was obtained, in accordance with the tenets of the Declaration of Helsinki. The protocol for this study was approved by the local Ethics Commission of the University of Bern in Switzerland (reference number: 152/08). Half of the patients from Lindenhofspital were from a previous clinical study on cytokine levels in AMD patients.[28]

2.8 | Cytokine analysis

Blood was collected and centrifuged for 15 minutes at 3000 *g*. Within 4 hours of the blood drawing, aliquots of serum were frozen at −20°C and stored at this temperature for up to 2 months, thereafter, at −80°C until the time of analysis, which was conducted simultaneously for all samples.

The Bio-Plex multiplex immunoassay beads system (Bio-Plex 100 array reader and Bio-Plex Manager software, version 6.1, Bio-Rad, Hercules, CA, USA) was used to simultaneously quantify the concentrations of 43 cytokines and chemokines according to the manufacturer instructions, as previously described.[28-32] A concentration standard was run in parallel on each test plate. It represented the average of triplicate standard dilutions of each corresponding chemokine/cytokine. A standard curve was generated and the sample concentrations were determined by curve-fitting. The assays were performed in a masked manner by an experienced technician.

2.9 | Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM) or standard deviation (SD), as indicated in the figure legends. The differences between the experimental groups were analyzed using student's *t* test or in the case of mouse flow cytometry results by Wilcoxon signed-rank test.

To ascertain that the human cytokine data were normally distributed the Shapiro-Wilk test was applied. Since the data did not meet the criteria of a normal distribution, the non-parametric Kruskal-Wallis H-Test was applied for the inter-group comparisons.

To control for Type I errors, but at the same time without drastically increasing Type II errors, the Holm's correction was applied. The Holm's correction, also known as the sequentially rejective Bonferroni test, progressively adapts the threshold for rejecting the null hypotheses. As a first step, all

P values were ranked from small to large, with *k* being the number of hypotheses. In a second step, the lowest *P* value was compared to α/k. If the *P* value was lower, the null hypothesis was rejected, and the result was considered significant. The same procedure was applied at the remaining *k*-1 hypotheses, where the threshold of significance was set at $\alpha/(k\text{-}1)$. This procedure was repeated sequentially for each *P* value, until the selected *P* value was not smaller compared to the sequential threshold.

The statistical analyses were performed using the open source software R (RStudio, Inc; psych package, version 3.3.2) and SPSS (IBM SPSS Statistics, version 23.0, Armonk, NY, USA).[29,30]

Differences between the experimental groups were considered statistically significant, when the probability value, *P* was <.05, marked with an asterisk (*) or <.01, marked with double asterisk (⁑).

3 | RESULTS

3.1 | Accumulation of CD163(+) cells in experimental CNV

To investigate the expression of CD163 in experimental CNV in the context of age, we performed immunohistochemistry (IHC). In the normal mouse retina and choroidal tissues from young and aged mice only very few CD163(+) macrophages were seen. In laser-injured eyes of young and aged mice a significant number of CD163(+) macrophages were seen in the CNV lesions (Figure 1A).

We performed western blotting using choroidal tissues from these animals, which showed a clear band for CD163 in laser-injured eyes in both young and aged mice (Figure 2A), in line with our prior report that CD163 was only expressed in the presence of CNV lesions, but was not higher in aged animals that did not have CNV.[18] In contrast to CD163, CD80 was not changed in aged mice or with CNV (Figure 2A).

Next, we performed laser-injury to Bruch's membrane in mice and collected peripheral blood for flow cytometry on day 7 after laser injury. When gating for monocytes, based on their characteristic forward and side scatters, the percentage of CD163(+) CD11b(+) was significantly higher in CNV mice, when compared with the unlasered controls (Figure 3A). In comparison, the percentage of CD80(+) CD11b(+) monocytes did not significantly change in the peripheral blood of CNV mice (Figure 3B).

We stained for CD163 and CD206 in the spleen, as it harbors a large portion of the body's monocytes within its red pulp.[33] Both CD163(+) and CD206(+) monocytes were found in the spleen of normal mice (Figure S1).

3.2 | Peripheral blood monocyte/macrophage markers in patients with AMD

Next, we examined the peripheral blood monocytes in patients with AMD and in demographically comparable

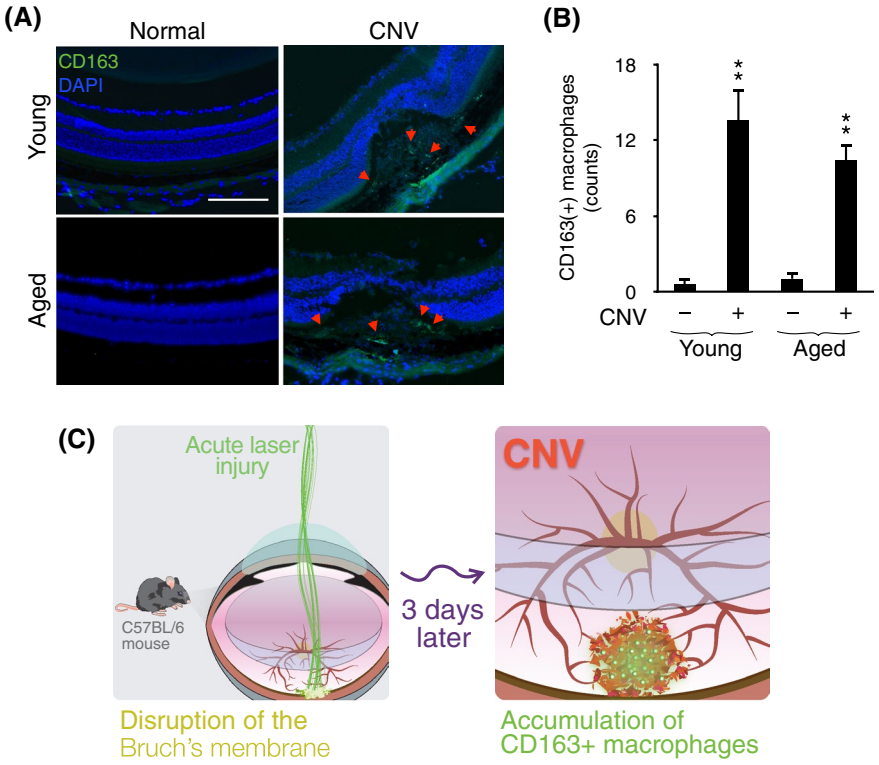

FIGURE 1 CD163(+) macrophages in experimental CNV. A, Representative immunohistochemistry for CD163 in normal and lasered WT mice and the corresponding quantifications. Blue, nuclei stained with 4,6-diamidino-2-phenylindole (DAPI). Arrows, CD163(+) macrophages. Bar, 50 μm. "⁑," $P < .01$ B, Quantification of the number of CD163(+) cells in histological sections from lasered mice (n = 6 in each group). C, Schematic of the acute laser-injury using short bursts of a 532 nm ophthalmologic laser to disrupt the Bruch's membrane. This leads to a robust choroidal neovascular response (CNV) and as we show here accumulation of CD163(+) cells in the laser-injured regions on day 3 post injury

healthy human subjects (Table 1). Blood samples were collected from patients with intermediate and advanced dry AMD (n = 34), wet AMD (n = 24), and control subjects (n = 28) and analyzed in flow cytometry.

When gated for CD68(+) cells with moderate CD163 expression, a significantly higher percentage of CD163(+) CD68(+) cells was found in peripheral blood of wet AMD ($P = .025$) and dry AMD ($P = .001$) in comparison to age-matched individuals who did not have AMD (Figure 4A). In contrast, the amount of CD80(+) CD68(+) and CD206(+) CD68(+) cells did not differ between patients with AMD and control subjects (Figure 4B).

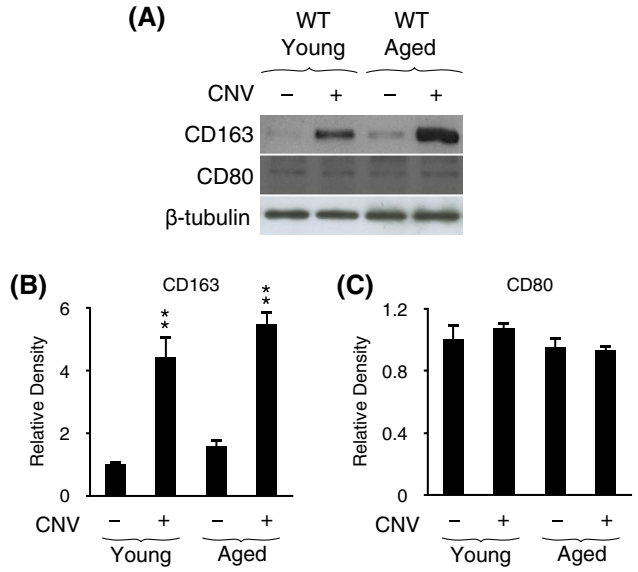

FIGURE 2 CD163 protein expression in experimental CNV. A, Representative western blots from whole cell lysates from choroids of young and aged mice with and without laser injury. B, Quantification of the western blots, n = 3 in each group, "⁂," $P < .01$

3.3 | Elevated sCD163 in patients with wet AMD

Next, we measured the amount of sCD163 in the serum of the various groups (Table 2). Representative fundus images from patients enrolled in our study illustrate their macula condition at the time of blood sampling (Figure 5A). Patients with wet AMD, both when exudative at the time of being blood drawn or not-exudative due to being treated with anti-VEGF intravitreal injections showed significantly higher sCD163 levels compared to the normal controls. The levels of sCD163 in patients with dry AMD showed the same trend, but did not reach statistical significance ($P > .05$) (Figure 5B).

3.4 | Peripheral blood cytokines in patients with AMD and normal subjects

A panel of 40 cytokines/chemokines were studied by immunoassay from serums of three groups of patients. Differences in the heat map between normal individuals, patients with dry AMD and patients with wet AMD were notable. There was an obvious distinction within the patients with wet AMD, some of whom showed a profile resembling that of the control individuals (low cytokine expressions), while others visibly differed from control (high cytokine expressions). This difference motivated us to separate the group of wet AMD patients into two subgroups, based on their clinical OCT images, one group in which patients showed active signs of exudation in OCT on the day of blood drawing (n = 8), while in the other group no signs of exudation were seen in OCT on the day of blood drawing (n = 8) (Figure 6).

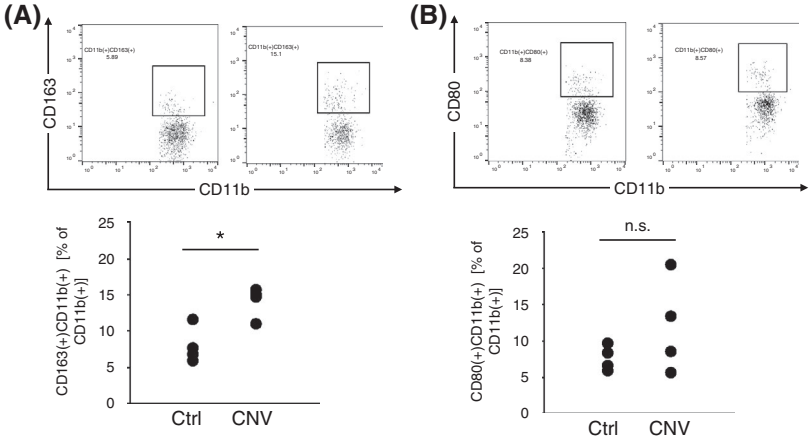

FIGURE 3 CD163(+) peripheral blood monocytes in experimental CNV. Peripheral blood of unlasered and laser-injured mice was collected on day 7 days after photocoagulation for FACS analysis. Up to 40 injuries were caused in each retina by a 532 nm diode laser (50 μm spot size, 100 ms duration, 100 mW) via a slit-lamp delivery system in a photocoagulator (Novus Verdi; Coherent Inc, Santa Clara, CA, USA). Peripheral blood monocytes were gated for by their characteristic forward and side scatters. The percentages of (A) CD11b/CD163 double positive and (B) CD11b/CD80 double positive cells were compared in lasered and unlasered mice. "*," $P < .05$

TABLE 1 Demographic data of the patients in the flow cytometry study

		Normal Control	Wet AMD	Dry AMD
Age in years (Mean ± SD)		70.9 ± 6.5	76.6 ± 5.4	73.4 ± 6.7
Gender, n (%)	Male	18 (64.3%)	16 (66.6%)	17 (50.0%)
	Female	10 (35.7%)	8 (33.4%)	17 (50.0%)
HTN, n (%)	No	20 (71.4%)	7 (29.1%)	13 (38.2%)
	Yes	8 (28.6%)	17 (70.8%)	21 (61.8%)
HLP, n (%)	No	26 (92.9%)	18 (75%)	23 (67.6%)
	Yes	2 (7.1%)	6 (25%)	11 (32.4%)
Smoking, n (%)	No	26 (92.9%)	21 (87.5%)	33 (97.1%)
	Yes	2 (7.1%)	3 (12.5%)	1 (2.9%)
Stage of Dry AMD[a]	Intermediate	n.a.	6 (12.5%)	51 (75%)
	Advanced	n.a.	2 (4.1%)	17 (25%)
CNV		n.a.	40 (83.3%)	n.a.
BCVA	OD	0.04 ± 0.09	0.91 ± 0.75	0.42 ± 0.37
	OS	0.08 ± 0.19	0.67 ± 0.46	0.33 ± 0.25

Abbreviations: BCVA, best-corrected visual acuity (Mean ± SD, logarithm of the minimal angle of resolution, logMAR); CNV, choroidal neovascularization; HLP: Hyperlipidemia; HTN, Hypertension; n.a., not applicable; OD, *oculus dexter*; OS, *oculus sinister*.

[a]Contralateral eye of the patient wet AMD.

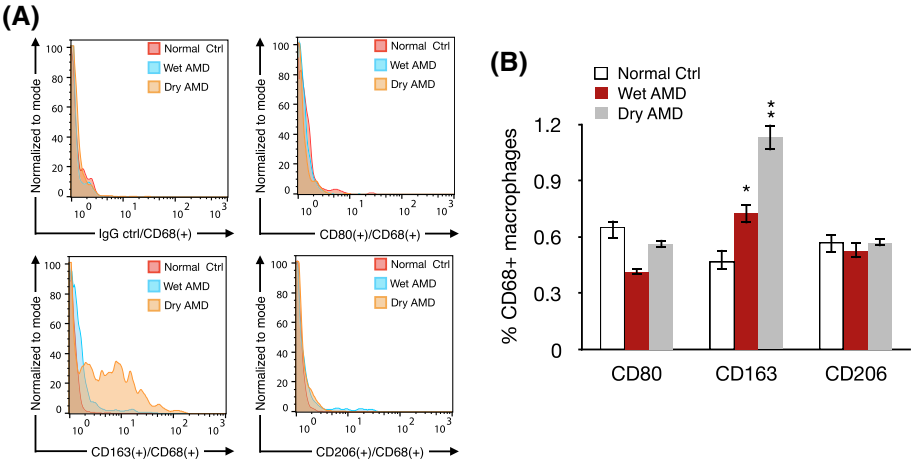

FIGURE 4 CD163(+) monocytes in the peripheral blood of normal individuals and patients with AMD. Flow cytometry analysis of peripheral blood from age-matched normal individuals, patients with dry, and wet AMD. CD68(+) monocytes were analyzed for CD80, CD163, and CD206 expression. A, Representative histograms of an age-matched normal subject, a patient with dry AMD and a patient with wet AMD. In the peripheral blood of the patient with dry AMD more CD163(+)/CD68(+) monocytes were found, while CD80(+)/CD68(+) monocytes did not differ between the normal subjects, patients with dry, and wet AMD. B, Quantitative analysis of the results of the peripheral blood flow cytometry; normal age-matched (n = 28), dry AMD (n = 34), and wet AMD (n = 24). "*," $P < .05$; "⁑," $P < .01$

Both groups were under anti-VEGF therapy, albeit with varying average durations between the last anti-VEGF therapy and the time of blood drawing. The patients with treated wet AMD, who showed dry maculae in OCT on the day of blood drawing were in average continuously treated with anti-VEGF (Ranibizumab [Lucentis, Novartis] or Aflibercept [Eylea, Bayer]) 8.8 ± 2.2 weeks prior to the time of blood drawing. In comparison, the patients with wet AMD, who showed subretinal and/or intraretinal fluid accumulation in OCT had had their last anti-VEGF treatment 7.0 ± 2.9 months before to the time of blood drawing, indicating the reactivation of a previously inactive CNV lesion several months after treatment cessation. The individual values for each patient and the statistical analysis are shown (Figure S2 and Tables S1 and S2). This finding is of clinical relevance, as it raises the possibility of determining or monitoring exudative disease activity in the patient's blood.

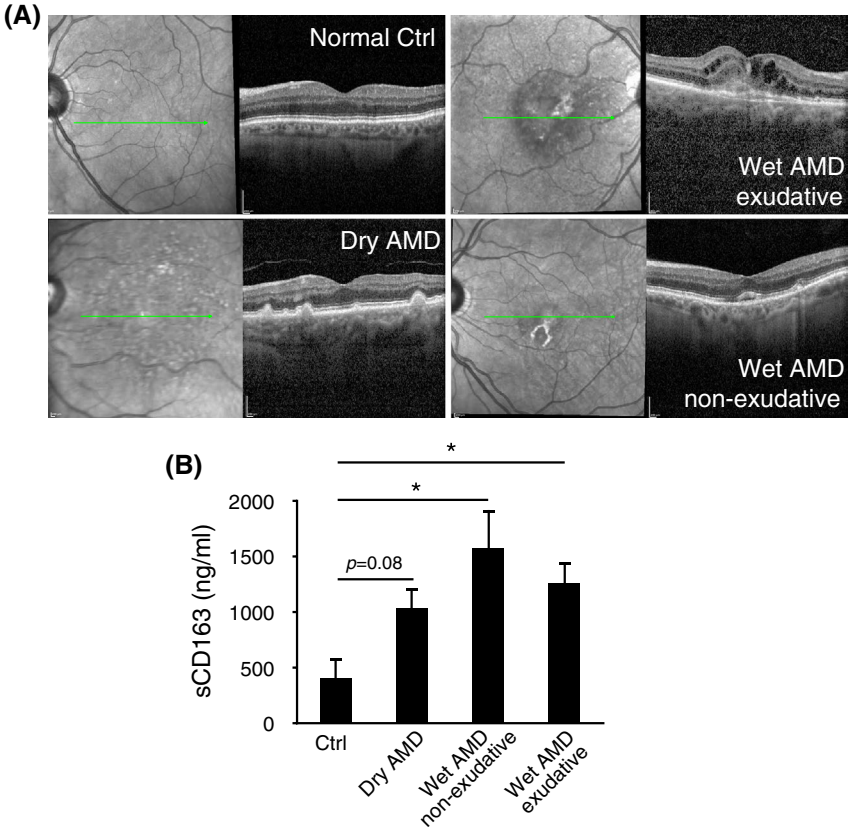

FIGURE 5 Soluble CD163 in the peripheral blood of normal individuals and patients with AMD. Peripheral blood samples from healthy individuals (n = 10), patients with dry (n = 12), and wet AMD (n = 16) were obtained, and sCD163 in the serum from these blood samples was measured by ELISA. Patients with wet AMD were divided into two groups, exudative at the time of blood drawing (n = 8) and non-exudative, as a result of successful prior anti-VEGF treatments (n = 8). A, Representative fundus reflectance scanning laser ophthalmoscopy (SLO) and OCT images from de-identified participant in each group are shown to illustrate the clinical condition on the day of blood sampling. B, Quantitative analysis of the sCD163 in the examined serum. "*," $P < .05$

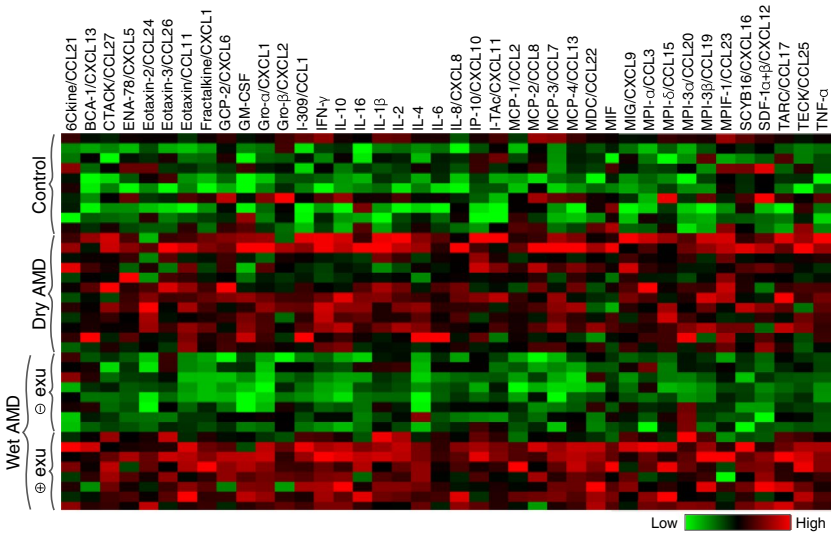

FIGURE 6 Peripheral blood cytokines in normal individuals and patients with AMD. A panel of 40 cytokines were measured in the serum samples, obtained from healthy individuals (n = 10), patients with dry (n = 12), and wet AMD (n = 16), using a multiplex immunoassay beads system (Bio-Plex). Heatmap results of the cytokine measurements. "⊕ exu," fundus examination showed signs of exudation; "⊖ exu" no sign of exudation

4 | DISCUSSION

The accumulation of macrophages in AMD lesions in the human eye was first shown in electron microscopy over three decades ago.[34] It has been discussed that macrophages may not only prevent, but also promote CNV formation.[35] However, only recently the possible role of CD163(+) M2-type macrophage in experimental CNV was reported.[18,36]

In this study, we examined the expression of CD163 in peripheral blood monocytes and soluble CD163 as a potential marker for AMD pathology and activity. In the young and old mouse eye, CD163(+) macrophages colocalized only with laser-injury. The CD80 protein expression did not differ between young and aged mice or with laser injury.

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of pathogenic processes.[37] There is currently no biomarker for early detection of neovascular AMD activity, before clinical signs such as leakage or vision loss occur. Killingsworth et al proposed that Drusen attract macrophages, which

TABLE 2 Demographic data of the patients in the serum-cytokine measurements

		Control	Dry AMD	Wet AMD (non-exudative)	Wet AMD (exudative)
Age in years (Mean ± SD)		70.2 ± 5.2	84.3 ± 6.4	83.4 ± 4.3	80.9 ± 3.7
Gender, n (%)	Female	8 (80%)	8 (67%)	5 (63%)	5 (63%)
	Male	2 (20%)	4 (33%)	3 (37%)	3 (37%)
HTN, n (%)	No	4 (40%)	6 (50%)	5 (63%)	5 (63%)
	Yes	6 (60%)	6 (50%)	3 (37%)	3 (37%)
HLP, n (%)	No	7 (70%)	11 (92%)	5 (63%)	7 (87%)
	Yes	3 (30%)	1 (8%)	3 (37%)	1 (13%)
Smoking, n (%)	No	8 (80%)	11 (92%)	8 (100%)	8 (100%)
	Yes	2 (20%)	1 (8%)	0 (0%)	0 (0%)
BCVA	OD	0.09 ± 0.13	0.32 ± 0.18	0.62 ± 0.19	0.80 ± 0.25
	OS	0.10 ± 0.18	0.28 ± 0.24	0.70 ± 0.24	0.76 ± 0.23

Abbreviations: BCVA: Best-corrected visual acuity (Mean ± SD, logarithm of the minimal angle of resolution, logMAR); HLP, Hyperlipidemia; HTN, Hypertension; OD, *oculus dexter*; OS, *oculus sinister*.

compromise Bruch's membrane and lead to neovascular lesions.[34] However, subretinal Drusen do not consistently and strongly associate with the clinical progression in AMD. This makes Drusen per se not a reliable biomarker for AMD.

Peripheral blood markers are of interest due to ease of access, the quantitative nature of the measurements, and the precision and reproducibility of the technologies involved. Flow cytometric analysis of peripheral blood immune cells is an established procedure that can be repeated longitudinally in individuals. An altered CD163(+) peripheral blood monocyte level was reported in acute myocardial infarction.[38] Our results showed intriguing differences between normal age-matched control subjects and AMD patients in the percentages of the CD163(+) cells and serum sCD163. The quantitative nature of the CD163 measurements in the peripheral blood holds promise to support other methods of AMD risk assessment for individual patients.

We found a higher percentage of CD163(+) CD68(+) to total numbers of CD68(+) monocytes in patients with dry AMD and wet AMD compared to non-AMD age-matched individuals. Patients with dry AMD had a higher percentage of CD163(+) monocytes in their peripheral blood than either age-matched controls or patients with wet AMD. A possible interpretation of the lower amount of CD163(+) monocytes in peripheral blood of patients with wet AMD in comparison to patients with dry AMD could be a higher amount margination of these cells, which would reduce their numbers in the peripheral blood. Alternatively, this difference could be owed to the impact of neutralizing VEGF in treated exudative AMD, compared to the untreated dry AMD patients.

Previously, more CD11b(+) peripheral blood neutrophils was reported in wet AMD patients.[39] Singh et al found CD200 expression on circulating CD11b(+) monocytes in wet AMD patients.[40] Analogous to tumor-associated M2 macrophages that correlate with the prognosis in cancer,[41] peripheral blood CD163(+) monocytes could in the future provide prognostic information in patients with AMD. This hypothesis however would need to be examined in a longitudinal prospective study of patients with early AMD, in whom the temporal dynamics of CD163(+) monocytes as well as sCD163 are assessed in conjunction with the risk of developing exudative AMD due to a CNV.

Liu et al found elevated levels of the chemokines, CXCL10, CCL14, CXCL16, CXCL7, and CCL22, in aqueous humor of patients with wet AMD, compared to those with dry AMD.[42] Others showed elevated levels of chemokine ligands CCL2, CCL5, CCL3, and CXCL10 in peripheral blood monocytes and also CCR1 and CCR2 on the CD14(+)CD16(+) monocyte population in patients with wet AMD.[43]

We found several cytokines to be higher in patients with wet AMD, who had shown exudative signs at the time of blood drawing, compared to healthy controls. However, these cytokines were not elevated in the patients with wet AMD, who did not show exudative signs at the time of blood drawing. This suggests that a vascular exudative activity of the lesions could potentially be monitored in the peripheral blood immune profile of the patient. The present study, however, does not address, whether the differences in peripheral blood cytokines in the various groups are mechanistically related to CD163(+) peripheral blood monocytes.

A key question that remains to be explored in future studies is whether the exudative switch in AMD is triggered by systemic disposition and roots, such as in the innate immune switch. If so, macrophages' high level of plasticity could open the door to immune-based therapy.

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DISCLOSURES

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

J.G. Garweg, H. Ahmadieh, and A. Hafezi-Moghadam designed research; N. Daftarian, S. Zandi, G. Piryaie, M. Nikougoftar Zarif, E. Ranaei Pirmardan, M. Yamaguchi, Q. Behzadian Nejad, H. Hasanpour, S. Samiei, I.B. Pfister, Z.-S. Soheili, and S. Nakao, performed research and analyzed data; N. Daftarian, A. Barakat, H. Ahmadieh, and A. Hafezi-Moghadam wrote the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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