IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Effect of Inhibition of Colony-Stimulating Factor 1 Receptor on Choroidal Neovascularization in Mice

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Neovascular age-related macular degeneration is one of the leading causes of blindness. Microglia and macrophages play a critical role in choroidal neovascularization (CNV) and may, therefore, be potential targets to modulate the disease course. This study evaluated the effect of the colony-stimulating factor-1 receptor inhibitor PLX5622 on experimental laser-induced CNV. A 98% reduction of retinal microglia cells was observed in the retina 1 week after initiation of PLX5622 treatment, preventing accumulation of macrophages within the laser site and leading to a reduction of leukocytes within the choroid after CNV induction. Mice treated with PLX5622 had a significantly faster decrease of the CNV lesion size, as revealed by *in vivo* imaging and immunohistochemistry from day 3 to day 14 compared with untreated mice. Several inflammatory modulators, such as chemokine (C-C motif) ligand 9, granulocyte-macrophage colony-stimulating factor, soluble tumor necrosis factor receptor-I, IL-1 α , and matrix metalloproteinase-2, were elevated in the acute phase of the disease when microglia were ablated with PLX5622, whereas other cytokines (eg, interferon- γ , IL-4, and IL-10) were reduced. Our results suggest that colony-stimulating factor-1 receptor inhibition may be a novel therapeutic target in patients with neovascular age-related macular degeneration. (*Am J Pathol* 2020, 190: 412–425; <https://doi.org/10.1016/j.ajpath.2019.10.011>)

The main feature of choroidal neovascularization (CNV) is new abnormal blood vessels emerging from the choroid and growing through the Bruch membrane and sometimes the retinal pigment epithelium (RPE). CNV is observed during neovascular age-related macular degeneration (AMD) and can lead to vision loss in patients experiencing AMD.^{1,2} Although the particular pathogenesis of AMD remains unknown, there are several previous studies showing the involvement of the innate immune system in the development of the disease.^{3–5}

Microglia, residing in the retina, are dynamic surveillants of the extracellular environment,⁶ located mainly in the ganglion cell layer, the inner nuclear layer, and the outer plexiform and nuclear layers. They can become reactive on injury and acquire migration and proliferation capabilities.⁷ Several studies have suggested that along with resident microglia, infiltrating macrophages may also play a

prominent role in the pathogenesis of AMD.^{8–11} In AMD, microglia/macrophages have been found in the subretinal space, where they are associated with drusen accumulation and CNV.¹² In experimental laser-induced CNV, macrophage depletion, using clodronate liposomes, resulted in reduced CNV size^{13,14}; and other studies have shown that the CNV area is significantly reduced in mice knocked out for the C-C chemokine receptor type 2 (*Ccr2*), a crucial

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component mediating macrophage infiltration.¹⁵ CCR2 as well as the CX3C chemokine receptor 1 are considered important factors for recruitment of macrophages to areas of inflammation and for the trafficking and the cellular migration of microglia into the subretinal space.¹² There is increasing evidence for their role in AMD development as lower expression of CX3C chemokine receptor 1 has been observed in patients with AMD.¹⁶

The involvement of microglia/macrophages in CNV is further supported by studies showing that reduction of immune cells' reactivity by intravitreal injections of polysialic acid leads to reduced vascular leakage in a laser-induced CNV mouse model.^{17,18} Moreover, loss of interferon- β and transforming growth factor- β signaling in retinal microglia has been implicated in increased microglia reactivity and exacerbated CNV lesions in mice,^{19,20} supporting a role of microglia cytokine signaling in the course of CNV.

Microglia depletion has been proved useful for the investigation of their involvement in several central nervous system disease paradigms,^{21,22} and it has been recently shown that brain microglia can be effectively eliminated by colony-stimulating factor-1 receptor (CSF-1R) inhibition.^{23,24} This was also observed in the retina, where microglia cells were eliminated by 92% to 97% in mice kept on a CSF-1R inhibitor-supplied diet for 1 week,^{25,26} whereas immune cells in the spleen and the bone marrow were not affected after 1 to 3 weeks of PLX5622 treatment.^{27–29} However, PLX5622-dependent reduction of antigen-presenting cells in the blood has been reported.²⁹ Cessation of CSF-1R inhibitor resulted in repopulation of retinal microglia from the remaining resident microglia pool in a CX3C chemokine receptor 1-dependent manner.^{30,31} However, more recent studies using bone marrow chimera mice suggest that monocyte-derived macrophages repopulate the retina, after cessation of PLX5622 treatment, where they adopt a ramified microglia-like morphology.³² Herein, the CSF-1R inhibitor PLX5622 was used during the whole duration of the experiments to gain insight in the role of retinal microglia/macrophages on the course of experimental laser-induced CNV.

Materials and Methods

Animals

This study was approved by the local Animal Ethics Committee (Veterinärdienst des Kantons Bern: BE 136/16) and conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Female mice were used, on the basis of the observation that female mice are more prone to CNV formation.³³ C57BL/6J mice (Charles River Laboratories, Sulzfeld, Germany) and MacGreen

[B6N.Cg-Tg(Csf1r-EGFP)1Hume/J]³⁴ heterozygous female mice (6 to 8 weeks old) were employed.

Mice had ad libitum access to PLX5622-containing chow (1200 parts per million formulated in AIN-76A standard rodent diet; Research Diets, Inc., New Brunswick, NJ) or AIN-76A standard rodent diet (control chow). Animals were housed, in groups of two to five, under temperature- and humidity-controlled conditions in individually ventilated cages with a 12-hour light/12-hour dark cycle. Before laser treatment or imaging, mice were anesthetized with 1 mg/kg medetomidine (Dormitor, 1 mg/mL; Provet AG, Lyssach, Switzerland) and 80 mg/kg ketamine (Ketalar, 50 mg/mL; Parke-Davis, Zurich, Switzerland), as previously described.²⁵ At the end of the intervention, medetomidine was antagonized by injection of 2.25 mg/kg atipamezole (Antisedan, 5 mg/mL; Provet AG). Before and 1 week after the initiation of the PLX5622 diet, as well as at days 3, 7, and 14 after laser treatment, mouse retinas were examined using confocal laser scanning ophthalmoscopy, fundus autofluorescence, and fluorescein angiography (Heidelberg Spectralis HRA 2; Heidelberg Engineering GmbH, Heidelberg, Germany). Groups of mice were euthanized with carbon dioxide inhalation at days 3 and 7 after laser treatment, and their retinas and choroid-RPE complexes were prepared for fluorescence-based flow cytometry or retina and choroid-RPE whole mounts and histology. All experiments were repeated at least once.

Laser-Induced Choroidal Neovascularization

Mice were anesthetized, and laser coagulation was performed using a 532-nm argon laser (Visulas 532s; Carl Zeiss Meditec AG, Oberkochen, Germany) with a slit-lamp adapter (Iridex Corp., Mountain View, CA) mounted on a slit lamp (BM900; Haag-Streit AG, Koeniz, Switzerland). Pupil dilation was achieved with tropicamide 0.5% and phenylephrine 2.5% eyedrops (Hospital Pharmacy, Inselspital, Bern, Switzerland). Hydroxypropyl methylcellulose, 20 mg/mL (Methocel 2%; OmniVision AG, Neuhausen, Switzerland), was applied on the eyes to keep them hydrated. A 2-mm fundus lens (Ocular Instruments, Inc., Bellevue, WA) was used for fundus visualization during the laser application. Three spots per eye were applied around the optic nerve, avoiding the large vessels (50- μ m size, 300-mW intensity, 100-millisecond duration), and both eyes were lasered per mouse. Rupture of Bruch membrane was indicated by bubble formation directly after laser application. Lesions with obvious hemorrhage were excluded from analysis. Bridging between laser spots was avoided by positioning the laser sites with sufficient distance.

Fundus Autofluorescence Imaging

Mice were anesthetized, and their pupils were dilated as described above. To avoid drying of the cornea with

resulting impairment of image quality, hydroxypropyl methylcellulose, 20 mg/mL, was applied on each eye. Retinal images were acquired using an ultrawide field 102-degree lens (Heidelberg Engineering GmbH), as described previously.³⁵ In MacGreen mice, green fluorescent protein (GFP)—positive cells (microglia/macrophages) could be visualized as hyperfluorescent spots in the autofluorescence images.

Fluorescein Angiography

After induction of anesthesia and pupil dilation (see above), 50 μ L of 0.01% fluorescein [Faure; Novartis, Basel, Switzerland; in 1 $\times$ phosphate buffered saline (PBS)] was administered subcutaneously; and images were acquired using the HRA system angiography (Heidelberg Spectralis HRA 2) with a noncontact ultrawide field 102-degree lens (Heidelberg Engineering GmbH). Images were taken during the first 90 seconds. CNV area was marked and measured using the caliper function in the Heidelberg Eye Explorer version 1.9.17.0 by two blinded assessors (D.K. and P.S.) (Heidelberg Engineering GmbH).

Immunohistochemistry Studies

For immunohistochemical studies, mouse eyes were isolated for preparation of retinal and choroid-RPE whole mounts. Eyes were fixed in 4% paraformaldehyde solution (pH 7.4) for 10 minutes, the anterior segments were removed (cornea and lens), and the posterior segment was incubated for another 50 minutes in paraformaldehyde solution. Retinas were mechanically detached from the choroid-RPE complex, and both tissues were extensively washed in 1 $\times$ PBS and 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and processed according to Ebnetter et al.³⁶ Isolectin GS-IB4 from Griffonia simplicifolia (Alexa Fluor 647 conjugate; 1:100; Thermo Fisher Scientific, Waltham, MA), a rabbit polyclonal antibody against ionized calcium-binding adapter molecule 1 (Iba-1; 1:500; Wako Pure Chemical Industries Ltd., Osaka, Japan), and a chicken polyclonal antibody against GFP (1:300; Abcam, Cambridge, UK) were used for labeling of blood vessels and microglia/macrophages. The secondary antibody goat anti-rabbit IgG H + L (Alexa Fluor 594 conjugate; 1:200; Thermo Fisher Scientific) was used for the visualization of Iba-1 staining, and goat polyclonal antibody to chicken IgY H + L (FICH; 1:200; Abcam) was used for visualization of GFP staining.

Another group of mice was euthanized at days 3 or 7 after CNV induction, and their eyes were fixed in 4% paraformaldehyde solution (pH 7.4) overnight at 4°C. Eyes were routinely embedded in paraffin, and paraffin sections (5 μ m thick) were cut running through the optic nerve head. The slides were deparaffinized and blocked with 10% normal goat serum for 30 minutes before incubation with a rabbit polyclonal antibody against Iba-1 (see

above) overnight at 4°C. The slides were washed in 1 $\times$ PBS and incubated with a secondary biotinylated goat anti-rabbit IgG antibody (1:250; Vector Laboratories, Burlingame, CA), for 30 minutes in room temperature, followed by three washes with 1 $\times$ PBS and incubation with a horseradish peroxidase–streptavidin conjugate (1:1000; Vector Laboratories) for 60 minutes at room temperature. The signal was visualized using the NOVA red substrate kit (Vector Laboratories), according to manufacturer's instructions.

Microscopy

For retinal or choroid-RPE whole mounts, microscopy was performed on equipment provided by the Microscopy Imaging Center, University of Bern (Bern, Switzerland). Retinal and choroid-RPE flat mounts were examined using an inverted Zeiss LSM 710 fluorescence confocal microscope (Carl Zeiss Meditec AG, Jena, Germany). Z-stacks of 100 to 110 μ m with 5- μ m intervals were obtained. Eye sections were examined under a fluorescence Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). CorelDraw X6 (Corel Corp., Ottawa, ON, Canada) was used for figure preparation.

Flow Cytometry

Retinas and choroid-RPE complexes from MacGreen mice were used for flow cytometry analysis at days 3 and 7 after the laser application. The eyes were collected in PBS (pH 7.4), the anterior segment was removed, the retina was mechanically detached from the choroid-RPE complex, and both tissues were used for flow cytometry. Retinas or choroid-RPE of each individual mouse were analyzed as one sample. Tissues were processed according to Ebnetter et al.³⁶ Retinas were stained with fluorescent-labeled antibodies against CD45-allophycocyanin/Cy7 (30-F11; 1:400), CD11b-allophycocyanin (M1/70; 1:200), and major histocompatibility complex class-II—Pacific blue (AF6-120.1; 1:200). Choroid-RPE samples were stained with fluorescent-labeled antibodies against CD11b-allophycocyanin (M1/70; 1:200), CD11c-allophycocyanin/Cy7 (N418; 1:200), Ly6G-PerCP/Cy5.5 (1A8; 1:200), Ly6C—Brilliant Violet 405 (HK1.4; 1:100), NK-1.1—phycoerythrin/Dazzle 594 (PK146; 1:200), CD3-phycoerythrin/Dazzle 594 (17A2; 1:200), and CD19-phycoerythrin/Dazzle 594 (6D5; 1:200). Zombie Red Fixable Viability Kit (1:800; Biolegend, San Diego, CA) staining was used for detection of dead cells, according to manufacturer's instructions. Samples were incubated for 20 minutes with an Fc blocker (1:200; Biolegend), followed by incubation with the fluorescent-labeled antibodies for 20 more minutes at 4°C in the dark. Each experiment was repeated at least once.

An LSR II Cytometer System with the BD FACSDiva software version 4.1 (BD Biosciences, Allschwil, Switzerland) was used for data acquisition. The flow cytometry data were analyzed with the Flowjo Single Cell

Analysis Software version 10 (TreeStar, Ashland, OR). All antibodies were purchased from Biolegend.

Protein Extraction

In total, 34 C57BL/6J mice were used for protein extraction and analysis of cytokine and chemokine levels. Control or PLX5622-fed mice were lasered, as mentioned above, 1 week after the initiation of the PLX5622 diet; and proteins were extracted from the posterior part of the eyes at days 3 and 7 after CNV. Ten naïve mice were used as naïve controls, and four eyecups were pooled as one sample. Briefly, the eyecups were homogenized in 200 μ L of lysis buffer [$1\times$ lysis buffer provided with the kit, 1 mmol/L Na_3VO_4 , protease inhibitor cocktail (cOmplete ULTRA Tablets, EDTA-free; Roche, Basel, Switzerland)] and homogenized using a Precellys 24 tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). After centrifugation for 10 minutes at $15,870 \times g$ at 4°C , the supernatant was collected and Bradford assay was used for the determination of total protein content.

Mouse Inflammation Antibody Array Membranes

Mouse inflammation antibody array membranes (ab193660; Abcam) were processed according to manufacturer's instructions. Briefly, the membranes were incubated for 2 hours in blocking buffer (provided with the kit) and incubated with 500 μ g of total protein, overnight at 4°C . Membranes were washed (wash buffer provided with the kit) and incubated with biotin-conjugated anti-cytokines/chemokines (provided with the kit) in blocking buffer, overnight at 4°C , and incubated with $1\times$ horseradish peroxidase-conjugated streptavidin (provided with the kit), for 2 hours at room temperature. Chemiluminescence was detected using detection buffers (provided with the kit) and a Fusion Pulse Imaging System (Witec AG, Luzern, Switzerland). Densitometry analysis was performed using the Protein Array Analyzer function of the ImageJ version 1.52h (NIH, Bethesda, MD; <https://imagej.net/imagej>).³⁷ The signal was normalized between different membranes using the positive control spots. Spots with abnormally high background were excluded from the analysis. The experiment was repeated once.

Statistical Analysis

The sample size was estimated in the GPower 3.1 software³⁸ on the basis of a power of 0.8 (80%) and a significance level of 0.05 (5%). The standardized difference of each experimental group was estimated on the basis of previous studies and pilot experiments.^{25,26} D'Agostino-Pearson omnibus test or Kolmogorov-Smirnov test was used to test the normal distribution of different data sets. Repeated-measures one-way analysis of variance was used for the analysis of the CNV course with or without

PLX5622 treatment. To compare the CNV area between PLX5622-treated and nontreated mice, ordinary one-way analysis of variance was used, followed by Tukey post hoc analysis. Statistically significant differences of the flow cytometry data were determined using one-way analysis of variance, followed by Tukey post hoc analysis for normally distributed data and Kruskal-Wallis test, followed by Dunn multiple comparison test for the data that did not follow normal distribution. Kruskal-Wallis test, followed by Dunn multiple comparison test, was used for the comparison of the array membrane data. All data are expressed as means $\pm$ SD.

$P < 0.05$ was considered statistically significant. GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA) was used for the statistical analysis.

Results

Effect of CSF-1R Inhibition in the Time Course of Laser-Induced CNV

Inhibition of the CSF-1R with PLX5622 for 7 days before CNV induction (Figure 1A) led to a striking reduction of GFP-positive cells in the retinas of MacGreen mice (Figure 1B). Laser application resulted in comparable CNV areas between PLX5622-treated and untreated mice 3 days after the CNV induction (Figure 1C). However, the area of CNV was decreased at later time points in the CNV group (Figure 1C), whereas the presence of PLX5622 resulted in an accelerated involution of CNV area over time (Figure 1C). The leakage area of CNVs on the fluorescein angiographs substantiated this finding ($P < 0.001$, repeated-measures one-way analysis of variance with Tukey post hoc analysis) (Figure 1D). Moreover, at day 14, there was a statistically significant decrease in CNV size of mice fed with PLX5622 compared with control-fed mice ($P < 0.001$, ordinary one-way analysis of variance with Tukey post hoc analysis).

In the choroid-RPE complex, CNV areas were identified using isolectin GS-IB4 staining (Figure 2A). Iba-1-positive cells representing microglia/macrophages accumulated around the lesion sites in the choroid-RPE and retinal whole mounts of control mice (Figure 2A). In the PLX5622 group, Iba-1-positive cells, most probably representing macrophages, were present in CNV areas of the choroid-RPE but were absent from the neurosensory retina (Figure 2A). In the choroid-RPE as well as in the outer retina, Iba-1-positive cells' morphology suggested a reactive phenotype, whereas in the inner retina, most of the Iba-1-positive cells had a ramified morphology (Figure 2B). Iba-1-positive cells were also detected in eye sections around the lasered area of CNV-subjected mice, and their numbers were greatly reduced in the presence of PLX5622 (Figure 2C). In the choroid-RPE complex of MacGreen CNV-subjected mice, most CSF-1R-GFP-positive cells were colocalized with Iba-1, whereas a few cells were Iba-1 negative (Figure 2D).

Figure 1 Effect of colony-stimulating factor-1 receptor inhibition on choroidal neovascularization (CNV). **A:** Experimental setup. **B:** Representative autofluorescence images of a MacGreen mouse retina before (PLX5622 day -7) and 7 days after the start of PLX5622 diet (PLX5622 day 0). Green fluorescent protein–positive microglia cells are drastically diminished in the retina after 7 days of PLX5622 diet. **C:** Representative fluorescein angiographs of CNV-subjected eyes at different time points in control or PLX5622-fed mice. **D:** CNV area measurements in fluorescein angiographs of control or PLX5622-fed mice. CNV area was gradually decreased in control (CNV) and PLX5622-fed mice. Repeated-measures one-way analysis of variance, followed by Tukey post hoc analysis, was used. Statistically significant reduction of the lesion size was observed in the presence of PLX5622 at day 14 compared with the control. Ordinary one-way analysis of variance, followed by Tukey post hoc analysis, was used. Individual CNV lesions are plotted in the graph. $n = 3$ (**B**); $n \geq 14$ eyes per group (**D**). *** $P < 0.001$.

Quantification of Retinal Microglia and Macrophage Populations with Flow Cytometry

Flow cytometry was performed at days 3 and 7 after CNV in the retina of control and PLX5622-treated CNV-subjected mice (Figure 3A). The cells were gated as shown in Supplemental Figure S1A. CNV induced accumulation of CD45^{low}CD11b⁺ microglia and CD45^{hi}CD11b⁺ macrophages³⁹ in the retina, whereas PLX5622 reduced their numbers (Figure 3A). Quantification of different cell populations revealed that the number of microglia and macrophages was elevated 3 and 7 days after CNV ($P < 0.001$, ordinary one-way analysis of variance with Tukey post hoc analysis) (Figure 3B). In mice fed with PLX5622, microglia cells were diminished by approximately 98.5% compared with control CNV mice at day 3 and day 7 after CNV ($P < 0.001$, ordinary one-way analysis of variance with Tukey post hoc analysis) (Figure 3B). Similarly, PLX5622 led to a reduction of macrophages by 47.7% and 76.3% compared with control at days 3 and 7, respectively, after CNV induction ($P < 0.001$, ordinary one-way analysis of variance with Tukey post hoc analysis) (Figure 3B). No

statistically significant difference was observed in CSF-1R expression in different cell types for different treatments and time points (Figure 3C). Increased major histocompatibility complex class-II expression in both microglia and macrophages was observed at day 3 after CNV ($P < 0.001$, Kruskal-Wallis test, followed by Dunn multiple comparison test) (Figure 3C). In mice fed with PLX5622, major histocompatibility complex class-II expression in microglia and macrophages was virtually absent (Figure 3C).

Quantification of Immune Cell Population in the Choroid-RPE with Flow Cytometry

Flow cytometry was performed at days 3 and 7 after CNV in the choroid-RPE of control and PLX5622-treated mice. The cells were gated as shown in Supplemental Figure S1B. The numbers of CD11c⁺ cells, most of which most probably represents dendritic cells,⁴⁰ leukocytes (CD11b⁺CD11c^{neg}),⁴¹ and neutrophils (CD11b⁺Ly6G⁺)⁴⁰ were elevated in the choroid-RPE during the acute phase of CNV ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively; ordinary one-way

Figure 2 *Ex vivo* evaluation of microglia and accumulation of monocyte-derived macrophages in the laser site. **A:** Isolectin and ionized calcium-binding adapter molecule 1 (Iba-1) staining in the choroid–retinal pigment epithelium (RPE) whole mounts and Iba-1 staining in retinal whole mounts 14 days after choroidal neovascularization (CNV) in control and PLX5622-fed mice. Iba-1–positive cells accumulate at the laser site in the choroid-RPE and in the retina of control CNV-subjected mice. In PLX5622-fed mice, Iba-1–positive cells are detected in the choroid-RPE but not in the retinal whole mounts. **Numbers** indicate the three different laser spots; **asterisks**, the optic nerve head; **yellow lines**, the area of the whole mounts. **B:** Higher magnification of Iba-1 staining in control-fed mice subjected to CNV, 14 days after the laser application. In the choroid-RPE and the outer retina, Iba-1–positive cells accumulated around the laser site and their morphology is characterized by increased soma size and retracted processes. In the inner retina, a ramified morphology is observed. **C:** Representative photomicrographs of Iba-1–positive cells in retinal sections of control or PLX5622-fed CNV-subjected mice. Iba-1–positive cells accumulated in the outer retina and the choroid-RPE in the CNV eyes (**first** and **second panels**). In mice fed with PLX5622, Iba-1–positive cells also accumulated in the outer retina and choroid-RPE; however, they were fewer in number and were almost absent 7 days after CNV (**third** and **fourth panels**). **D:** Representative photomicrographs of Iba-1– and colony-stimulating factor-1 receptor–green fluorescent protein (CSF-1R–GFP)–positive cells in retinal sections of control or PLX5622-fed mice 14 days after CNV. Most of the CSF-1R–GFP–positive cells are colocalized with Iba-1, whereas a few of them are Iba-1 negative (**arrows**). $n \geq 6$ mice per group (**A**); $n \geq 3$ mice per time point (**C** and **D**). Scale bars: 500 μm (**A**); 200 μm (**B** and **C**); 100 μm (**D**). Original magnification: $\times 10$ (**A**); $\times 40$ (**B** and **D**); $\times 20$ (**C**). IPL, inner plexiform layer; ONL, outer nuclear layer.

Figure 3 Flow cytometry analysis of microglia/macrophage population in the retina of choroidal neovascularization (CNV)-subjected mice. **A:** Representative flow cytometry plots at different time points after CNV in control and PLX5622-fed animals. Microglia were identified as CD45^{low} CD11b⁺ and macrophages as CD45^{hi} CD11b⁺. **B:** Quantification of microglia (CD45^{low} CD11b⁺; **top panel**) and macrophages (CD45^{hi} CD11b⁺; **bottom panel**) in the retina, 3 and 7 days after CNV, in the presence or absence of PLX5622. Elevated number of microglia and macrophages is observed 3 and 7 days after CNV. PLX5622 reduces the number of microglia and macrophages in the retina. **C:** Quantification of colony-stimulating factor-1 receptor-positive (CSF-1R⁺) or major histocompatibility complex class-II-positive (MHC-II⁺) microglia (**left**) and CSF-1R⁺ or MHC-II⁺ macrophages (**right**), expressed as a percentage of the total number of microglia or macrophages, respectively. No statistically significant difference was observed in the number of CSF-1R⁺ microglia and macrophages in the presence or absence of PLX5622. MHC-II⁺ microglia and macrophages were detected in the retina at the early course of CNV (day 3), yet this increase was not statistically significant compared with the naive tissue. PLX5622 reduces the expression of MHC-II by these cells. One-way analysis of variance, followed by Tukey post hoc analysis, or Kruskal-Wallis test, followed by Dunn multiple comparison test, was used. $n \geq 5$ mice per group (**B** and **C**). *** $P < 0.001$.

analysis of variance with Tukey post hoc analysis for CD11c⁺ cells and leukocytes and Kruskal-Wallis test, followed by Dunn multiple comparison test, for neutrophils) (**Figure 4A**), whereas PLX5622 led to a reduction of CD11c⁺ cells and leukocyte numbers below control (naive) levels (**Figure 4A**). Interestingly, although there was no difference between the naive and the CNV-subjected tissues in the total number of Ly6G^{neg} Ly6C^{low/neg} side scatter (SSC-H)^{low} cells, representing nonclassic patrolling monocytes/macrophages,⁴²

the presence of PLX5622 led to a reduction of these cell numbers below control (naive) levels ($P < 0.001$, ordinary one-way analysis of variance with Tukey post hoc analysis) (**Figure 4B**). Classic inflammatory monocytes/macrophages (Ly6G^{neg} Ly6C^{hi}),⁴² on the other hand, were increased at day 3 after CNV; and this was prevented by PLX5622 ($P < 0.001$, ordinary one-way analysis of variance with Tukey post hoc analysis) (**Figure 4B**). The expression of CSF-1R by patrolling and inflammatory monocytes,

Figure 4 Quantification of immune cell population in the choroid-retinal pigment epithelium (RPE) using flow cytometry analysis.

A: The numbers of CD11c⁺ cells (left) in the choroid-RPE are elevated 3 days after choroidal neovascularization (CNV) and return to control levels at day 7 after CNV. PLX5622 (PLX) reduces CD11c⁺ cell numbers below control levels. Leukocyte numbers (middle) peak at day 3 after CNV, and their numbers are reduced in the presence of PLX5622. A slight increase in neutrophil numbers is detected 3 days after CNV (right). **B:** Leukocytes were further gated on the basis of the expression of Ly6G and Ly6C. Ly6G^{neg} Ly6C^{low/neg} side scatter (SSC)-H^{low} cells are reduced in the presence of PLX5622, whereas no differences are observed between the naïve and CNV-subjected tissues (left). On the other hand, Ly6G^{neg} Ly6C^{hi} inflammatory monocytes/macrophages are increased 3 days after CNV and decreased thereafter (right); and this phenomenon is prevented by PLX5622. **C:** No difference is observed in the expression of colony-stimulating factor-1 receptor (CSF-1R) by Ly6G^{neg} Ly6C^{low/neg} SSC-H^{low} (left) or Ly6G^{neg} Ly6C^{hi} monocytes/macrophages (right) between different treatments. Ordinary one-way analysis of variance with Tukey post hoc analysis or Kruskal-Wallis test, followed by Dunn multiple comparison test, was used. $n \geq 5$ for each treatment (A–C). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

expressed as a percentage of Ly6G^{neg} Ly6C^{low/neg} SSC-H^{low} and Ly6G^{neg} Ly6C^{hi} cells, respectively, was not altered between the different groups (Figure 4C).

Cytokine Levels during the Course of CNV

The protein levels of several inflammatory modulators, such as chemokines [chemokine (C-C motif) ligands 3 and 9], growth factors [insulin-like growth factor-binding protein (IGFBP)-3, IGFBP-5, and granulocyte-macrophage

colony-stimulating factor], members of the tumor necrosis factor (TNF) superfamily [CD30 ligand and soluble TNF receptor (TNFR) I], inflammatory cytokines (interferon- γ , IL-1 α , and IL-12 p70), anti-inflammatory cytokines (IL-13), matrix metalloproteinases (MMP-2 and MMP-3), Fc γ receptors (Fc- γ RIIB), and adhesion molecules [vascular cell adhesion molecule 1 (VCAM-1)], were elevated at the acute phase of the disease (Table 1) 3 days after laser application. During the later phase (day 7), most of the inflammatory modulators returned to control levels, except for an

Table 1 Effect of Laser-Induced CNV on Cytokine/Chemokine Levels in the C57BL/6J Mouse Eyecups in the Presence or Absence of PLX5622

Factors	Day 3		Day 7	
	CNV	CNV + PLX5622	CNV	CNV + PLX5622
Chemokines				
CCL3	1.60*	1.11	0.85 ^{‡‡}	0.55
CCL9	1.58***	2.36*** ^{†††}	1.26	2.62*** ^{†††}
CX3CL1	1.34	4.59***	1.39	3.75***
CXCL2	1.19	1.46***	1.08	1.39** [†]
CXCL13	1.34	2.12*** [†]	1.06	1.46
CXCL16	1.29	2.38*** ^{†††}	1.11	2.45*** ^{†††}
Growth factors				
bFGF	1.67	2.06*	2.12***	3.00*** [†]
IGFBP-3	1.33**	1.83*** ^{†††}	1.08	1.70*** ^{†††}
IGFBP-5	1.45*	2.41*** ^{†††}	1.12	1.92*** ^{†††}
IGFBP-6	1.13	2.80** [†]	1.32	1.84
GM-CSF	1.68*	2.89*** ^{†††}	1.66*	1.91** ^{‡‡}
M-CSF	1.12	1.33*	1.15	1.02
TNF superfamily				
CD30	1.21	2.03** [†]	1.09	1.54
CD30L	1.43*	1.40	1.09	0.92
Fas ligand	0.79	1.75 ^{††}	0.85	0.93
sTNFR1	1.53*	2.58*** ^{†††}	0.86 ^{‡‡}	2.35*** ^{†††}
Proinflammatory cytokines				
IFN- γ	1.70*	0.54 ^{†††}	1.14 [‡]	0.46 [†]
IL-1 α	1.34*	2.31*** ^{†††}	1.12	1.60*** ^{†††††}
IL-12 p70	1.48**	1.76***	1.09 [‡]	1.50*
TCA-3	1.20	1.86*** ^{†††}	0.95	1.73*** ^{†††}
TIMP-1 α	1.23	1.59**	0.93	1.41 [†]
Anti-inflammatory cytokines				
IL-4	1.06	2.72 ^{†††}	0.89	2.75 ^{†††}
IL-10	1.40	3.13*** ^{†††}	1.53	2.00* [‡]
IL-13	1.45**	1.35	1.08 [†]	0.84 [‡]
Tissue remodeling				
MMP-2	1.86***	5.07*** ^{†††}	0.84 [‡]	5.62*** ^{†††}
MMP-3	1.59*	1.85**	1.13	2.29*** ^{†††}
Fc-γ receptors				
Fc γ RIIB	2.19**	0.43 ^{††}	1.45	0.89
Adhesion molecules				
VCAM-1	1.54**	2.84*** ^{†††}	1.57***	3.47*** ^{†††}

Semiquantitative analysis of inflammatory mediators' protein levels in the retina—choroid—retinal pigment epithelium complex, 3 and 7 days after CNV, in the presence or absence of PLX5622. All data are normalized to naive tissues. Only targets with statistically significant differences are shown in the table. Targets that were examined but did not show statistically significant differences included IL-6, TNF- α , vascular endothelial growth factor, monocyte chemoattractant protein 1 (MCP-1), G-CSF, and CCL5, among others.

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus naive.

[†] $P < 0.05$, ^{††} $P < 0.01$, and ^{†††} $P < 0.001$ versus CNV.

[‡] $P < 0.05$, ^{‡‡} $P < 0.01$, and ^{‡‡‡} $P < 0.001$ versus the same treatment at day 3.

bFGF, basic fibroblast growth factor; CD30L, CD30 ligand; CCL, chemokine (C-C motif) ligand; CNV, choroidal neovascularization; CX3CL-1, chemokine (C-X3-C motif) ligand 1; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN- γ , interferon- γ ; IGFBP, insulin-like growth factor-binding protein; MMP, matrix metalloproteinase; sTNFR, soluble TNF receptor; TCA-3, chemokine (C-C motif) ligand 1 or CCL1; TIMP1, TIMP metalloproteinase inhibitor 1; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

elevation of the protein levels of basic fibroblast growth factor, granulocyte-macrophage colony-stimulating factor, and VCAM-1. In the presence of PLX5622, protein levels of several targets were elevated (Table 1), in agreement with previous studies,⁴³ suggesting that PLX5622 actions are not mediated by a general reduction of the overall inflammatory response.

Discussion

Accumulation of microglia/macrophages in the subretinal space of CNV lesions and expression of inflammatory cytokines have both been implicated in the formation of CNV.^{10,12,44,45} However, the role of microglia/macrophages in CNV development is not clear yet because contradictory

roles of these cells have been reported. Previous studies have shown that macrophage depletion can lead to a reduction of vascular endothelial growth factor (VEGF) production and CNV lesion size in mice,^{13,14} whereas other studies reported that lack of the CCR2 receptor and its ligand chemokine (C-C motif) ligand 2 in mice leads to spontaneous CNV development after 9 months of age.² Moreover, microglia accumulation has been observed in AMD lesions in chemokine (C-C motif) ligand 2/CX3C chemokine receptor 1-deficient mice but not in control mice,⁴⁶ whereas several additional studies reported that resident microglia actively contribute to CNV development.^{12,47,48} Microglia/macrophage involvement in CNV is further supported by studies showing that reduction of microglia and macrophage reactivity by polysialic acid or translocator protein 18-kDa ligands leads to reduced neurodegeneration and less vascular leakage in a CNV mouse model.^{17,49,50}

Herein, we show that CSF-1R inhibition with PLX5622 leads to faster involution of CNV, as revealed by *in vivo* imaging and immunohistochemical studies in retinal and choroid-RPE whole mounts (Figures 1 and 2). PLX5622 efficiently depletes retinal microglia as early as 1 week of treatment (Figures 1–3). The flow cytometry data showed that PLX5622 reduced the total number of CD45^{hi}CD11b⁺ cells in the retina, most probably representing invading monocyte-derived macrophages and perivascular macrophages, and largely prevented major histocompatibility complex class-II expression by microglia and CD45^{hi}CD11b⁺ cells (Figure 3C). The effect of PLX5622 was not observed on CSF-1R expression by microglia or macrophages (Figure 3C). Recent studies by Paschalis et al³² suggested that ocular injury could trigger infiltration of peripheral monocytes into the retina, where they adopt a ramified morphology, similar to resting microglia, and express low levels of CSF-1R.⁵¹ However, this was not observed in the present study.

Flow cytometry analysis was also performed in the choroid-RPE complex of CNV-subjected mice fed with control or PLX5622 chow. These data revealed increased numbers of CD11c⁺ cells, leukocytes, and neutrophils after CNV (Figure 4), which is in agreement with previous studies.^{52,53} CD11c⁺ dendritic cells have been previously reported to play a role in CNV development. Specifically, they have been found to accumulate in the CNV lesions, peaking in numbers between day 2 and 4 after laser-induced CNV in mice.⁵³ Moreover, i.v. injection of dendritic cells leads to their accumulation into the CNV lesions, where they are associated with increased CNV size.⁵³ Our data are consistent with these findings. Moreover, inhibition of the CSF-1R with PLX5622 did not only deplete retinal microglia, but it also affected the number of CD11c⁺ cells and leukocytes found in the choroid-RPE by reducing their numbers below naïve levels; it did not have any effect on neutrophil numbers (Figure 4A). Previous studies have shown that CSF-1R signaling is vital for dendritic cell

differentiation, and dendritic cells are diminished in the spleen and peritoneum of CSF-1R-deficient mice.⁵⁴ Moreover, *Csf1r* depletion has been recently shown to negatively regulate the dendritic cell pool size in adult mice.⁵⁵

Further analysis of leukocyte subsets showed an increase of inflammatory classic monocytes/macrophages (Ly6C^{neg}/Ly6C^{hi}) 3 days after CNV, which was prevented by PLX5622. However, the population of patrolling nonclassic monocytes/macrophages in the choroid-RPE, identified as Ly6C^{neg}/Ly6C^{low/neg}/SSC-H^{low}, was not affected by laser-induced CNV, but PLX5622 reduced their number below naïve levels, whereas the remaining cells were still positive for CSF-1R expression (Figure 4B). This suggests that PLX5622 leads to a reduction of resident choroidal macrophage numbers and prevents the recruitment of Ly6C^{hi} inflammatory monocytes/macrophages into the affected area. Indeed, by using neutralizing antibodies against CSF-1R or its ligand CSF-1, it has been previously shown that blocking of CSF-1R signaling can lead to depletion of Ly6C^{neg} monocytes in the blood, whereas it only has modest effects in Ly6C⁺ monocytes in the bone marrow.^{56–58} Thus, the beneficial effects of PLX5622 may be attributed at least partially to the reduced numbers of peripheral immune cells, which, along with retinal microglia, could contribute to the extracellular matrix remodeling that triggers the formation of new blood vessels.

To further investigate the effect of PLX5622 in the course of CNV, a panel of cytokines/chemokines was analyzed in CNV-subjected mice fed with PLX5622 or normal food, using a semiquantitative assay (the array membrane data are openly available in OSF Data repository—Open Science Framework at <https://osf.io>; reference number: y5n7h). Elevated levels of several cytokines/chemokines were found in the CNV-subjected eyes, whereas some of them were elevated even more in the presence of PLX5622 (Table 1). Among the highest up-regulated cytokines were CX3CL1, MMP-2, basic fibroblast growth factor, and VCAM-1, whereas others, such as IL-6, TNF- α , and monocyte chemoattractant protein 1 (MCP-1), were not changed compared with naïve mice. CX3CL1 signaling is a key modulator of macrophage recruitment into the injured tissues,^{59,60} and it has been shown to reduce microglial activation and subsequent neurotoxicity.^{61,62} MMP-2, on the other hand, has been reported to play a prominent role in CNV formation because reduced CNV is observed in mice deficient for MMP-2.^{63–65} MMPs can proteolytically cleave several chemokines, leading to their inactivation or the generation of antagonistic derivatives, which cannot promote chemotaxis.⁶⁶

No differences were detected in the levels of vascular endothelial growth factor (VEGF) between the experimental groups 3 and 7 days after CNV induction. VEGF is considered the major proangiogenic factor in the inflamed retina,^{67–69} and it has been suggested that interaction between VEGF and other proangiogenic factors, such as basic fibroblast growth factor, is required for the angiogenic actions of VEGF.⁷⁰ Herein, an up-regulation of basic

fibroblast growth factor was observed in the presence or absence of PLX5622, but no differences were observed in VEGF protein levels between naïve and CNV mice in the presence or absence of PLX5622. Most likely, these differences could be observed in earlier time points after the CNV induction.

In the presence of PLX5622, the proinflammatory IL-1 α was also elevated at day 3 after CNV laser induction and it was significantly reduced at day 7. Up-regulation of the adhesion molecule VCAM-1 was also observed in our study, and this up-regulation was greater in the presence of PLX5622. VCAM-1 mediates the adhesion of monocyte-derived macrophages to vascular endothelial cells, and increased protein levels of VCAM-1 have been reported in the retinal vasculature during CNV in mice.¹¹ However, increased infiltration of monocyte-derived macrophages into the injured retina was not observed in the presence of PLX5622.

In addition, significantly elevated levels of soluble TNFRI were found during the disease course of CNV in the presence of PLX5622. After TNF- α binds to its membrane receptors, TNFRI and TNFRII, the receptors are cleaved to their soluble forms (soluble TNFRI/soluble TNFRII) by metalloproteinases.⁷¹ The level of surface expression of TNFRI and its soluble form was found to be an important factor in regulating TNF- α -mediated effects,^{72,73} and the receptor cleavage acts as an important mechanism for the suppression of TNF- α -mediated inflammation.⁷⁴ The levels of the anti-inflammatory cytokines IL-4 and IL-10 were also elevated in the PLX5622 group, and this was accompanied by down-regulation of interferon- γ levels. Indeed, IL-10 has been previously reported to inhibit the production of interferon- γ ⁷⁵ and to mediate a suppressive effect on CNV development.⁷⁶ In addition, IL-4 has been shown to antagonize interferon- γ -induced activities in macrophages.^{77–79} The expression of IGFBP-3, IGFBP-5, and IGFBP-6 was also increased in the presence of PLX5622. IGFBPs bind with high activity to IGF and thus limit the free form of IGF in the circulation,^{80–82} thereby acting as angiogenesis suppressors.^{83–85} In keeping with this, IGF signaling has been proposed to play a role in diabetic retinopathy and retinal neovascularization, where it has been shown to lead to increased VEGF levels.^{86–88}

Because of complex cytokine interactions that may act antagonistically, the apparent increase of the inflammatory cytokines after PLX5622, which is in keeping with previously published studies,⁴³ may ultimately lead to a more angiostatic/antiangiogenic phenotype and thus inhibit CNV progression. In addition, it has been previously reported that CSF-1 can increase the release of VEGF from monocytes and CSF-1R inhibition can lead to lower levels of VEGF.^{89,90} Thus, blockade of the receptor may affect the angiogenesis mediated by CSF-1R-expressing cells. Moreover, physical contact between immune cells, such as perivascular macrophages, and endothelial cells may be required for the progression of CNV. *In vitro* studies have

shown that direct contact between monocytes and endothelial cells can increase endothelial cell proliferation.^{48,91,92} Hence, reduction of retinal microglia and attenuation of perivascular macrophages could result in decreased endothelial cell proliferation and, therefore, regression of CNV lesions.

Our data are in agreement with previous studies that have shown elevation of cytokine and chemokine levels in the retinas of CNV-subjected mice, as well as infiltration of monocyte-derived macrophages into the retina early during the course of CNV.^{11,93–95} Apart from microglia and monocyte-derived macrophages, RPE cells and vascular endothelial cells are also known to produce cytokines during CNV in mice.⁹⁶ Moreover, activated Müller cells could also be a source of cytokines during CNV. On the basis of the PLX5622 depletion data, immune cells are likely not the main producers of cytokines during the course of our CNV model, yet they play an important role in CNV progression.

In summary, the present study highlights the important role of innate immunity in the course of CNV in mice. Inhibition of the CSF-1R has beneficial effects against CNV progression, but whether these effects are mediated by depletion of microglia and/or macrophages in the retina, by patrolling monocytes in the choroid, or by the prevention of leukocyte influx needs to be further investigated. In addition, a more detailed analysis of the cytokine and chemokine levels after PLX5622 treatment could provide valuable data toward specific biological effects of PLX5622 in retinal pathology.

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Author Contributions

P.S. designed and performed the experiments, analyzed and interpreted the data, and revised the manuscript; D.K. designed and performed the experiments, analyzed and interpreted the data, and wrote and revised the manuscript; A.E. contributed to the study design and revised the manuscript; M.S.Z. conceived and designed the experiments, interpreted the data, wrote and revised the manuscript, and supervised the project; all authors read and approved the final manuscript.

Supplemental Data

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