VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

IL-4 Regulates Specific Arg-1⁺ Macrophage sFlt-1—Mediated Inhibition of Angiogenesis

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One of the main drivers for neovascularization in age-related macular degeneration is activation of innate immunity in the presence of macrophages. Here, we demonstrate that T helper cell type 2 cytokines and, in particular, IL-4 condition human and murine monocyte phenotype toward Arg-1⁺, and their subsequent behavior limits angiogenesis by increasing soluble fms-like tyrosine kinase 1 (sFlt-1) gene expression. We document that T helper cell type 2 cytokine-conditioned murine macrophages neutralize vascular endothelial growth factor-mediated endothelial cell proliferation (human umbilical vein endothelial cell and choroidal vasculature) in a sFlt-1—dependent manner. We demonstrate that *in vivo* intravitreal administration of IL-4 attenuates laser-induced choroidal neovascularization (L-CNV) due to specific IL-4 conditioning of macrophages. IL-4 induces the expression of sFlt-1 by resident CD11b⁺ retinal microglia and infiltrating myeloid cells but not from retinal pigment epithelium. IL-4—induced suppression of L-CNV is not prevented when sFlt-1 expression is attenuated in retinal pigment epithelium. IL-4—mediated suppression of L-CNV was abrogated in IL-4R—deficient mice and in bone marrow chimeras reconstituted with myeloid cells that had undergone lentiviral-mediated shRNA silencing of sFlt-1, demonstrating the critical role of this cell population. Together, these data establish how IL-4 directly drives macrophage sFlt-1 production expressing an Arg-1⁺ phenotype and support the therapeutic potential of targeted IL-4 conditioning within the tissue to regulate disease conditions such as neovascular age-related macular degeneration. (*Am J Pathol* 2015, 185: 2324–2335; <http://dx.doi.org/10.1016/j.ajpath.2015.04.013>)

Age-related macular degeneration (AMD) is a leading cause of blindness in developed countries. Neovascular AMD (nAMD) leads to irreversible visual loss in 90% of patients¹ through subretinal hemorrhage, photoreceptor cell death, and, ultimately, irreversible fibrovascular scarring. The altered immune responses that accompany AMD are an attractive target for therapy that has long-term effects and that might complement current practice of neutralizing vascular endothelial growth factor (VEGF) or as currently investigated in clinical trials that target soluble fms-like tyrosine kinase 1 (sFlt-1) via gene therapy (registered at ClinicalTrials.gov as NCT01494805). Laser damage to the mouse retinal pigment epithelium (RPE) and Bruch membrane [laser-induced choroidal neovascularization (L-CNV)] simulates mechanisms of human nAMD and has amply demonstrated the utility of anti-VEGF

therapies.^{2,3} Although the model is not a true model of nAMD because the condition in animals follows an acute wound-healing tempo rather than the more insidious pathologic changes and low-grade changes to immune responses observed in AMD and specifically nAMD, it nevertheless was used, for example, to unravel the controversy over the role of IL-18 and

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the inflammasome in CNV.⁴ In this model, as in humans, L-CNV is accompanied by myeloid cell infiltration and activation,^{5–7} release of proinflammatory factors,^{6,8} and macrophage-mediated proangiogenic events,^{9,10} which we have shown are attenuated via specific suppression (via CD200R) of myeloid cell activation.¹¹ Thus, the concept of subverting the innate immune drive during AMD by resetting the threshold of myeloid activation or conditioning myeloid cell responses is an attractive therapeutic goal with promise to overcome longer term limitations of current successful antiangiogenic therapies. Rapid progress could be made by resetting myeloid activation thresholds, particularly because applications are currently using recombinant IL-4 in clinical trials (registered at [ClinicalTrials.gov](https://clinicaltrials.gov) as NCT00014677, NCT00003842, and NCT00000769) that will further illuminate the therapeutic potential for ocular neovascular disorders, such as AMD.

It is currently thought that macrophage phenotypes, such as classic (M1) and alternative (M2) activation,¹² generated by conditioning cells via T helper cell type 1 and type 2 (Th2) cytokine stimulation, respectively, have both distinct and overlapping cell functions. Th2 cytokine conditioning is commonly associated with asthma,¹³ psoriasis,¹⁴ and rheumatoid arthritis,¹⁵ where it leads to vessel formation and increased expression of VEGF and adhesion molecules. Our data,¹⁶ and that of others,^{7,17–19} indicate that cells with M1, M2, and mixed phenotypes can all contribute to the regulation of angiogenesis that is irrespective of phenotype signatures such as Arg-1 and Ym-1 of M2 cells. Therefore, distinct macrophage phenotypes per se do not dictate antiangiogenic or proangiogenic functions, and additional microenvironment stimuli regulate macrophage-mediated neovascularization. So in addition to their well-known proangiogenic VEGF-producing function,^{9,16,20} macrophages are also antiangiogenic under certain conditions [including, for example, hypoxia, interferon (IFN)- γ , and lipopolysaccharide (LPS)],^{21–23} and this plasticity provides opportunities to harness macrophages to promote antiangiogenic responses.

Conditioning macrophage behavior to express VEGF receptor 1 (VEGFR1; alias Flt-1), a decoy receptor that possesses greater VEGF-binding affinity than the angiogenic VEGFR2,^{24–26} may produce longer term attenuation of nAMD. Such a notion is supported by several lines of evidence. Derangement of sFlt-1 homeostasis has pathologic implications,²⁷ and levels of sFlt-1 act as a biomarker of neovascular diseases.²⁸ Blocking VEGF/VEGFR1 interactions in macrophages reduce monocyte tumor necrosis factor- α and IL-6 production in rheumatoid arthritis^{29,30}; VEGFR1-deficient mice display abnormal vascular development and die *in utero*,³¹ whereas deletion of Flt-1 tyrosine kinase domains is nonlethal.²⁴ sFlt-1 retains high affinity for VEGF³² and regulates VEGF activity, for example, sustaining corneal avascularity.³³ Exogenous sFlt-1 was exploited³⁴ to inhibit angiogenesis in animal models of cancer³⁵ and atherosclerosis³⁶ and is in development of a phase 2 trial for nAMD, after successful preclinical studies.^{37,38} In the context of inducing remission,

resetting the tissue control of angiogenesis through the manipulation of macrophage phenotype has the potential to restore long-term tissue health.

We report here that antiangiogenic conditioning by the Th2 cytokine IL-4 induces myeloid cells to secrete sFlt-1 and to confer on them the ability to suppress angiogenesis. This myeloid cell-restricted response affords therapeutic opportunities to alter the retinal environment, reset myeloid activation responses, and suppress the development and/or persistence of CNV in humans.

Materials and Methods

Mice

C57BL/6 (*Cd45.2*) and SJL (*Cd45.1*) mice were obtained from Harlan UK Limited (Oxford, UK), and all breeding colonies were established within the Animal Service Unit at the University of Bristol (Bristol, United Kingdom). C57BL/6 Tg4^{IL-4R α -/-} (IL-4R^{-/-}) were a kind gift from Prof. David Wraith (University of Bristol, Bristol, United Kingdom). All animals were housed under specific pathogen-free conditions with continuously available food and water. Treatment of animals conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were performed in compliance with University of Bristol institutional guidelines and under the authority of project licenses from the UK Home Office.

Cell Culture

Generation of bone marrow-derived macrophages (BMDMs) followed the protocol from the original report.³⁹ In brief, mouse femurs and tibia were collected, and bone marrow cells were flushed out, followed by 8-day maturation in Teflon bags (supplied by Dr. M. Munder, University of Heidelberg, Heidelberg, Germany) with Dulbecco's modified Eagle's medium (DMEM) that contained 10% heat-inactivated fetal calf serum (FCS), 5% normal horse serum, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 U/mL penicillin-streptomycin (all from PAA Laboratories, Pasching, Austria), 50 mmol/L 2-mercaptoethanol (Life Technologies, Paisley, United Kingdom), and 50 pg/mL macrophage colony-stimulating factor generated from L929 fibroblast-conditioned media. Cells were then harvested and washed by DMEM before stimulation. BMDMs (1×10^6 /mL) were treated with IL-4, IL-13 (20 U/mL), IL-10 (8 U/mL; all from PeproTech, London, United Kingdom), IFN- γ (100 U/mL), or LPS (055 B5; 100 ng/mL; both from Sigma-Aldrich, St. Louis, MO) 4 hours in complete media (DMEM that contained 10% FCS, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 100 U/mL penicillin-streptomycin) for gene expression assessed by quantitative real-time RT-PCR (RT-qPCR) or 72 hours for protein expression detected by enzyme-linked immunosorbent assay

(ELISA). Concentration and period of stimulation varied as defined in [Results](#).

For experiments that used human cells, peripheral blood mononuclear cells were isolated from whole blood by using Ficoll-Paque PLUS (GE Healthcare, Cardiff, United Kingdom), followed by magnetically activated cell sorted-positive beads (Miltenyi Biotec, Surrey, United Kingdom) for the enrichment of CD14⁺ monocytes. A concentration of 1×10^6 /mL CD14⁺ cells were cultured in 48-well plates with media alone or with recombinant human IL-4 (5 or 10 ng/mL; BioLegend, San Diego, CA) for 4 and 48 hours in complete media (RPMI that contained 2 mmol/L L-glutamine and 100 U/mL penicillin-streptomycin) for gene expression assessed by RT-qPCR or protein expression detected by ELISA.

Primary RPE cell culture was generated from trypsinized C57BL/6 mouse choroid tissue. Briefly, eyes were kept on ice for 3 hours before dissection for choroid eye cups, followed by incubation with 0.25% trypsin (PAA Laboratories) in 37°C for 1.5 hours. The RPE layer was carefully scrapped by sterile scalpel in DMEM that contained 20% FCS and 100 U/mL penicillin-streptomycin. Disrupted tissue was then passed through 21- and 23-gauge needles to make a single-cell suspension, followed by two washes in DMEM. Cells from each eye were seeded in 2 mL of Hanks' balanced salt solution (PAA Laboratories) with 20% FCS, 2 mmol/L L-glutamine, and 100 U/mL penicillin-streptomycin for 14 days, and fresh media was replaced every 3 to 4 days. Gene expression for primary RPE cells was assessed by RT-qPCR after 20 U/mL of IL-4 stimulation for 4 hours, or sFlt-1 production was measured by ELISA on supernatant fluid collected from cells treated for 48 hours with prostaglandin E₂ (PGE₂) and/or IL-4, LPS, or IFN- γ .

ELISA for VEGF and sFlt-1 and Binding Assay

Pan VEGF-A (denoted as VEGF) and sFlt-1 in murine and human culture media (CM) were detected by ELISA, following the manufacturer's direction (R&D Systems, Oxford, United Kingdom). VEGF/sFlt-1 binding assay was designed to measure the capacity of BMDM-derived sFlt-1 to bind VEGF₁₆₄. Briefly, 0.2 μ g/mL VEGF₁₆₄ capture antibody was coated onto Nunc immunoplates (VWR, Lutterworth, United Kingdom) overnight at 4°C, followed by washes with 0.5% Tween 20 (Sigma-Aldrich) in phosphate-buffered saline (PBS). Recombinant VEGF₁₆₄ (200 pg/mL) or just 1% phosphate-buffered saline albumin was incubated for 1 hour at room temperature, and then plates were washed three times. After this, BMDM-conditioned media or recombinant sFlt-1 (200 ng/mL) was applied to the wells for 1 hour of incubation at room temperature. After three washes, 1 μ g/mL biotinylated anti-sFlt-1 detection antibody was added to the wells for 1 hour at room temperature, and the plates were washed. ExtrAvidin-Peroxidase (100 μ L; dilution 1:1000 in PBS; Sigma-Aldrich) was applied for 30 minutes at room temperature, plates were washed, and the 3,3',5,5'-tetramethylbenzidine substrate (BD Biosciences, Oxford,

United Kingdom) was added. The reaction was terminated by adding 2N H₂SO₄ before optical density of each well was determined as the degree of sFlt-1 to VEGF binding.

Quantitative Reverse Transcription-PCR

After stimulation, cells were washed with PBS and lyzed in TRIzol reagent (Life Technologies), and total mRNA was isolated. Total RNA (1 μ g) dissolved in RNase-free water (Life Technologies) was used for reverse transcription by ImProm-II Reverse Transcriptase (Promega, Madison, WI), following the manufacturer's instructions. For gene analysis of rare cell populations, CD11b⁺-sorted retinal microglia, lentiviral-transduced green fluorescent protein (GFP⁺) splenic macrophages or BMDM (at least 6000 cells per sample) was achieved with Cell-to-Ct kit (Ambion Life Technologies, Austin, TX).

Target gene detection was performed with Power SYBR Green PCR master Mix (Applied Biosystems, Foster City, CA) with specific mouse primer pairs of *Vegfa*, *Arg1*,⁴⁰ *Chil3* (Ym1),⁴¹ *Nos2*, and *Gapdh* (RTPrimerDB database ID:3483 and ID:2920, respectively) obtained from the literature, and specific full-length Flt-1 (forward: 5'-GCCCGGGCCTTCAATAAAATA-3'; reverse: 5'-CTTTTTC-CCGCAGTGCTC-3'), *sFlt-1* (forward: 5'-AGAAGAC-TCGGGCACCTATG-3'; reverse: 5'-AGGTTTTGAAG-CAGGTGTGG-3'), and *Il-10* (forward: 5'-TGAATTCC-CTGGGTGAGAAGCTGA-3'; reverse: 5'-TGGCCTTGT-AGACACCTTGGTCTT-3') were designed by Primer3-Web online tool (<http://primer3.sourceforge.net>, last accessed April 17, 2015). Human-specific primer pairs of sFlt-1 (forward: 5'-GGCTGTTTTCTCTCGGATCTC-3'; reverse: 5'-CATCTCCTCCGAGCCTGAAAG-3')⁴² and the sequences for *18S* (forward: 5'-CACGGACAGGAT-TGACAGATT-3'; reverse: 5'-GCCAGAGTCTCGTTCTG-TTATC-3') were designed by Primer3Web online tool. For gene expression analysis, the relative quantification method was used with normalization to *Gapdh* or *18S*, in mouse or human samples, respectively.

Fluorescence-Activated Cell Sorting Analysis

Mouse tail blood samples were erythrocyte-lyzed by ACK (ammonium-chloride-potassium) buffer and Fc blocked by CD16/32 antibody (dilution 1:100; clone: 93, eBioscience, San Diego, CA). Cells were subsequently stained with Biotin-CD45.1 (clone A20), allophycocyanin-CD45.2 (clone 104), and allophycocyanin-cyanine 7-CD11b (clone: M1/70) antibodies (dilution 1:100; BD Pharmingen, Heidelberg, Germany), followed by incubation with Streptavidin-eFluor 450 (dilution 1:100; eBioscience). Fluorescence staining of cells was assessed with a BD LSRII system flow cytometry with FACS Diva software version 8.0 (BD Biosciences). Data were analyzed and presented with FlowJo software version 7.6.5 (TreeStar Inc., San Carlos, CA).

In Vitro sFlt-1 Knockdown in BMDMs

To knockdown BMDM sFlt-1 expression, specific siRNA (Stealth RNAi; Life Technologies) was generated, followed by transfection protocol modified from the manual. In brief, a mixed transfectant of knockdown siRNA or scrambled RNA 50 nmol/L and 2.4 μ L of Lipofectamine RNAiMAX (Life Technologies) in 150 μ L of Opti-MEM I Reduced Serum Medium (Life Technologies) was incubated for 30 minutes at room temperature before being subsequently mixed with 150 μ L of BMDMs (8×10^6 /mL). Cells were then seeded into 12-well plates and incubated for 4 hours, before replacement of the transfection media with 500 μ L of penicillin-streptomycin-free complete media for 16 hours of incubation. Additional stimuli (eg, IL-4 or IL-13) in 500 μ L of complete media (with antibiotics) were applied to the cultures and left for 72 hours before harvesting of the conditioned media.

HUVEC Proliferation Assay

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords donated from healthy pregnancies at St. Michael's Hospital (Bristol, UK) under local ethics committee approval with consent. Cells were cultured in EBM-2 with 100 U/mL penicillin-streptomycin and supplement kit EGM-2 SingleQuots (excluding VEGF supplementary vial; all from Lonza, Slough, United Kingdom) until confluent, and then cultures were split. For HUVEC proliferation assay, 25,000 cells were washed and seeded in 100 μ L of EBM-2 in a 96-well flat-bottom plate 16 hours before experimental assay. BMDM-conditioned media or recombinant sFlt-1 was mixed 1:1 (v/v) with 10 ng/mL of recombinant VEGF₁₆₄ (PeproTech) in EBM-2, supplemented with 10% FCS for 16 hours of incubation at 4°C. Seeding media were discarded, and 50 μ L of assay mixture was applied to HUVECs in each well for incubation at 37°C. After 4 days of culture, AlamarBlue (Serotec, Oxford, United Kingdom) was used to measure end point cell viability, following manufacturer's instructions, and degree of cell proliferation was revealed by hematoxylin staining.

Choroidal Explant Assay

The peripheral choroid-scleral complex was dissected from the eyes of B10.RIII mice aged 6 to 8 weeks old and cut into approximately 1-mm $\times$ 1-mm pieces before Matrigel seeding in 48-well plates (BD Biosciences). The Matrigel explants were cultured in 0.4 mL of endothelial growth medium media (Promocell, Heidelberg, Germany) that contained 5% FCS, with or without recombinant 12 ng/mL sFlt-1, and media were replaced on days 3 and 5. On day 7, phase-contrast images of explants were obtained with a Widefield imaging system microscope (Leica, Milton Keynes, United Kingdom). Depending on the extent of sprouting, on occasion composite images (comprising several images) of explants were prepared. The area of

angiogenic sprouting quantified by using ImageJ 1.46r (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).

In Vivo sFlt-1 Knockdown and Myeloid Chimeric Mice

To generate lentivirus that expressed shRNA that targeted *sFlt-1*, the scrambled sequence (GTCGTTCTTTATACCGTGAAACAAT) or sequences for sFlt-1-shRNA (CCACACAAAGTCATGTCAAACATTA) were cloned into GFP-containing plasmid pLL3.7⁴³ [originally obtained from Addgene (Cambridge, MA) plasmid 11,795], following designer's cloning protocol with 5' XhoI overhang and loop sequences (TTCAAGAGA). Cloned plasmids and packing plasmids, pMDG-VSVG and p8.91, were transfected to 293T cells by polyethylenimine for lentivirus production, followed by purification as previously described,⁴⁴ and infectious viral titer was checked.

To knockdown sFlt-1 in RPE *in vivo*, sFlt-1 shRNA or scrambled lentivirus were subretinally injected into the superior and inferior hemispheres of each eye, with successful injection confirmed by bullous retinal detachment. Mice were then left for 2 weeks before laser lesions were introduced for CNV. For sFlt-1 knockdown in peripheral myeloid cells, bone marrow chimera mice were generated. In brief, bone marrow cells from a C57BL/6 CD45.2 mouse were lineage depleted (Miltenyi Biotec, Surrey, United Kingdom) and preincubated in STIF media (Iscove's Modified Dulbecco's Media that contained 10% FCS, 50 ng/mL stem cell factor, 20 ng/mL thyroid peroxidase, 20 ng/mL insulinlike growth factor 2, and 10 ng/mL fibroblast growth factor, all recombinant proteins from PeproTech) at 1×10^6 /mL for 24 hours. Cells were then transferred to plates coated with RetroNectin (Takara, Kyoto, Japan) and mixed with lentivirus (MOI 3), followed by spinoculation at $800 \times g$ in room temperature for 1 hour. Cells were then incubated for 48 hours before harvested for fluorescence-activated cell sorting for GFP⁺ population. Each SJL CD45.1 mouse was lethally irradiated (1000 rad) and transferred intravenously with at least 5×10^4 GFP⁺ lentivirus-infected cells. Chimeric mice were left for 6 weeks to recover peripheral myeloid populations, and reconstitution was checked by flow cytometry on tail blood samples.

L-CNV

Mice were administered tropicamide (0.5% w/v; Bausch & Lomb, Aubenais, France) to dilate pupils and were intraperitoneally injected with 200 μ L of recovery anesthetic per mouse (6% Vetalar V; Pfizer Limited, Kent, United Kingdom; 10% Rompun; Bayer plc., Newbury, United Kingdom). Four laser spots were introduced in each eye onto posterior retina by using OculightSlx Krypton Red Laser system (power 200 mmol/L, during 75 milliseconds, spot size 75 μ m). PBS or IL-4 (1 μ L, 0.6 μ g/mL; BD Bioscience) were injected intravitreally with 33-gauge needle (Hamilton, Reno, NV) immediately and at day 7 after laser injury. Littermates were selected at random for

Figure 1 IL-4 or IL-13 stimulates sFlt-1 expression of BMDMs and cytokine conditioned CM perturbs detection of VEGF. **A:** VEGF expression is detected from supernatant fluid of BMDM stimulated with PGE₂ in the presence of 20 U/mL IL-4 or IL-13. **B:** An additional 200 pg/mL VEGF recombinant protein was incubated with various dilutions of macrophage CM preconditioned for 24 hours with 20 U/mL IL-4 or IL-13, and VEGF levels were quantified via ELISA. **C:** Dynamic mRNA expression from BMDMs after 4 hours of cytokine stimulation was analyzed by RT-qPCR. **D:** Soluble Flt-1 production by using cytokine-conditioned media was assessed by ELISA. **E:** BMDMs were stimulated with IL-4 or IL-13 in the presence of PGE₂ or IL-10 for 2 days, culture supernatant fluid was then assessed for sFlt-1 by ELISA and presented as an inhibition percentage (sFlt-1 conc. IL-4 only, 181.2 ± 23.45 ng/mL; IL-13 only, 143.1 ± 26.76 ng/mL). The data are expressed as means $\pm$ SEM. $n = 3$ individual experiments with triplicates. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus media alone with Mann-Whitney test (**A**, **C–E**) or one-way analysis of variance with Bonferroni post-test (**B**). BMDM, bone marrow-derived macrophage; CM, culture media; ELISA, enzyme-linked immunosorbent assay; PGE₂, prostaglandin E₂; RT-qPCR, quantitative real-time RT-PCR; sFlt-1, soluble fms-like tyrosine kinase 1; VEGF, vascular endothelial growth factor.

L-CNV and assigned to various intravitreal treatment groups. For CNV area analysis, mice were culled at day 7 or 14 after laser injury. The eyes were collected for fixation in 4% paraformaldehyde at 4°C overnight, followed by microdissection for choroidal tissue, which was permeabilized and blocked in PBST-1 (PBS that contained 1% bovine serum albumin and 0.5% Triton X-100; Sigma-Aldrich) at 4°C overnight. Choroids were then immunostained with 10 μ g/mL biotinylated IB4 (L2140; Sigma-Aldrich), 1 μ g/mL rabbit anti-mouse Iba-1 (019-19,741; Wako Chemicals GmbH, Neuss, Germany), and 1:100 dilution of rabbit anti-Collagen IV antibody (2150-1470; AbD Serotec, Oxford, United Kingdom), followed by staining with 2.5 μ g/mL Streptavidin-Rhodamine Red X (016-290-084; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and 7 μ g/mL Pacific Orange-goat anti-rabbit IgG (P31584, Life Technologies). Whole choroid tissue were flat-mounted in Vectashield (Vector Laboratories, Peterborough, United Kingdom), and CNV areas were examined from images obtained with a Leica TCS-SP2-AOBS confocal laser scanning microscope, followed by size measurement of IB4 reactive area by using ImageJ software version 1.46r. Measurement of the CNV area and analysis were blinded and processed by different investigators (J.L. and J.W.). Merged lesions and eyes with hemorrhage were excluded for analysis.

Statistical Analysis

Data were analyzed by one-way analysis of variance with Bonferroni test or the *U* test (GraphPad Instant software

version 4; GraphPad Inc., San Diego, CA) to assess differences between groups. Data are expressed as means $\pm$ SEM of at least three independent experiments, unless otherwise specified.

For *in vivo* L-CNV experiments, the null hypothesis was that there is no difference in disease incidence, mean maximum severity, day of onset, or type of immune cell-involved burden between groups (ie, no effect) and was tested with $P < 0.05$ as significant. Animal numbers are assessed such that the experiments are powered to a probability of 80% that a statistically significant result will be observed. To test the mean maximum severity (CNV lesion area) between groups, the nonparametric *U* test was used as non-Gaussian distribution.

Results

IL-4 or IL-13 Stimulate Macrophage sFlt-1 Expression and Inhibit Canonical PGE₂-Driven VEGF Production

Human CD14⁺CD16^{+/–} monocytes express IL-4R,⁴⁵ and we found that IL-4 treatment induced sFlt-1 gene expression and protein in human (Supplemental Figure S1) and murine monocytes. To determine whether it is possible to redress myeloid cell responses in ocular angiogenesis, we further interrogated mechanisms and therapeutic potential in the mouse. PGE₂ strongly stimulates VEGF and conditions macrophages to an *Arg-1*⁺ M2 phenotype.¹⁶ In contrast, M2-conditioning cytokines, IL-4 and IL-13, appeared to abrogate the amount of VEGF protein detected from

Figure 2 IL-4 or IL-13 incites release of free form of sFlt-1 and suppresses angiogenesis *in vitro*. **A:** Schematic of modified free form sFlt-1 detection assay. Immunosorbent signals were detected after incubation with or without VEGF (V) and recombinant sFlt-1 (F), normal macrophage CM (M), or media from macrophages conditioned with IL-4 (4) or IL-13 (13). **B:** Conditioned CM of cells alone, IL-4-, or IL-13-stimulated, was preincubated with 0.5 nmol/L VEGF, followed by application to HUVEC culture for 4 days. Kinetic HUVEC activity as demonstrated via AlamarBlue (AB) utilization over a 36-hour culture period where also increased numbers (proliferation) are corroborated via hematoxylin staining and bright field microscopy (left panel). **C:** Cytokine-free CM was generated from BMDMs preincubated with IL-4 or IL-13 for 2 to 18 hours (media collected as comparative with CM), and then cells were washed twice and reconstituted in culture with fresh media for up to 48 hours before collection (cytokine-free CM). Soluble Flt-1 concentration was quantified by ELISA. Results are presented as relative percentages of samples to control cytokine-induced 48-hour stimulation, and contributions of sFlt-1 in cytokine-free CM are shown in filled bars (sFlt-1 concentration = 188.21 ± 8.65 ng/mL and 140.22 ± 19.69 ng/mL for IL-4 and IL-13 conditioning, respectively). **D:** Influence of CM was examined further in HUVEC activity assay. **E:** BMDMs were transfected with specific siRNA, scrambled RNA, or OPTI-MEM (media) alone to silence sFlt-1 expression in the presence of IL-4 or IL-13 for 72 hours, and CM was assessed for sFlt-1 levels by ELISA. **F:** sFlt-1-mediated modulation of HUVEC activity. Data are expressed as means $\pm$ SEM. $n = 3$ individual experiments with triplicates. *** $P < 0.001$ versus V + M (A) or versus scrambled, with Mann-Whitney test (E) or one-way analysis of variance with Bonferroni post-test (B, D, F). Original magnification: $\times 2.5$. AB, antibody; BMDM, bone marrow-derived macrophage; CM, culture media; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; sFlt-1, soluble fms-like tyrosine kinase 1; VEGF, vascular endothelial growth factor.

PGE₂-treated BMDMs (Figure 1A). IL-4- or IL-13-conditioned BMDM CM inhibited the measurement of VEGF, suppressing its detection by >50% (Figure 1B), raising the possibility that IL-4 or IL-13 induces soluble factors that neutralize the bioactivity of VEGF measured by immunoassay. Consistent with this, Vegfa mRNA expression was not affected by IL-4 or IL-13 treatment, but both sFlt-1 and full-length Flt-1, the predominant VEGFR expressed by macrophages,⁴⁶ were up-regulated by IL-4 and IL-13 (Figure 1C and Supplemental Figure S2A). A significant increase of sFlt-1 protein was also observed in both IL-4 and IL-13 conditioned CM (Figure 1D), whereas conditioning with classical M1 signals such as LPS or IFN- γ failed to stimulate comparable levels of sFlt-1 (Supplemental Figure S2B). By contrast, PGE₂ dramatically suppressed detectable levels of sFlt-1 triggered by IL-4 or IL-13 (Figure 1E), implying either that a similar mechanism of VEGF binding to sFlt-1 could disturb detection of sFlt-1 in the ELISA or that PGE₂ could attenuate sFlt-1 mRNA production.

IL-4- or IL-13-Induced Macrophage sFlt-1 Expression Neutralizes VEGF-Mediated Angiogenesis *in Vitro*

To determine whether the observed VEGF inhibition could be explained by sFlt-1 binding to VEGF in CM we designed a binding assay (Figure 2A) that allowed detection of VEGF-sFlt-1 complexes induced, for example, by PGE₂ and IL-4. Both IL-4- and IL-13-stimulated monocytes produced sFlt-1 that bound to VEGF (Figure 2A). We reasoned that IL-4 or IL-13 CM may possess antiangiogenic activity that could be tested by VEGF-mediated HUVEC proliferation. VEGF alone in the presence of macrophage CM stimulated proliferation, but this was inhibited if CM came from macrophages treated with IL-4 or IL-13 (Figure 2B). Suppression of HUVEC activity was not due to contamination by IL-4 or IL-13 in the culture because CM collected after 48 hours from macrophages, treated for 6 hours, and then extensively washed contained 45% to 52% of the sFlt-1 measured in macrophage cultures treated for the full 48 hours (Figure 2C) and was able to suppress VEGF-induced HUVEC proliferation (Figure 2D). To confirm that sFlt-1 was responsible for this effect, we performed a sFlt-1 siRNA knockdown (Figure 2E) in macrophages treated with IL-4 or IL-13. Knockdown of sFlt-1 restored the proliferation of HUVECs in the presence of CM, further confirming that IL-4 or IL-13 promoted macrophage sFlt-1 expression to mediate endothelial growth (Figure 2F).

Because M1 or M2 macrophage phenotypes are reciprocally antagonistic,^{47,48} we asked whether IL-4- or IL-13-induced BMDM sFlt-1 expression was influenced by the M1-polarizing IFN- γ (Figure 3A) and LPS (Figure 3B). LPS or IFN- γ antagonized IL-4- or IL-13-mediated induction of *Arg-1*, the pan M2 phenotype marker, but we noted that both sFlt-1 and Flt-1 up-regulation in M2 conditions remained unaffected by additional IFN- γ or LPS. The data confirm that M2 macrophages derived by IL-4 or IL-13 conditioning secrete sFlt-1, and this function is not antagonized by subsequent M1

Figure 3 IFN- γ or LPS alters gene profile of M2 macrophage activation without change in *sFlt-1* expression. Bone marrow-derived macrophages stimulated with 20 U/mL IL-4 or IL-13 in the presence of varying concentrations of IFN- γ (A) or LPS (B) for 4 hours, followed by RT-qPCR analysis. ** $P < 0.01$, *** $P < 0.001$ versus no IFN- γ (A) or no LPS (B) with one-way analysis of variance with Bonferroni post-test. IFN, interferon; LPS, lipopolysaccharide; RT-qPCR, quantitative real-time RT-PCR.

conditioning. Consistent with our earlier report,¹⁶ these results further reinforce the assertion that M1 or M2 phenotype alone does not entirely determine functional outcome in promoting or regulating angiogenesis because macrophages integrate complex microenvironment and cytokine cues through time.⁴⁹

Although both Th2 cytokines drove sFlt-1 responses and induced macrophage-mediated inhibition on HUVEC proliferation, the effect of IL-4 was more pronounced. IL-4 and IL-13 trigger similar biological functions and signaling cascades,⁵⁰ and to demonstrate a requirement for signaling via the IL-4R we used BMDMs from IL-4 receptor-deficient mice and demonstrated that these cells failed to up-regulate sFlt-1 gene or protein expression when stimulated with recombinant IL-4 (Figure 4). Our subsequent experiments focused on the potential of IL-4 conditioning alone, in particular because rapid translation of these findings to human disease is possible, given current clinical use of rIL-4 in human trials for other indications.

Both Resident Microglia and Infiltrating Macrophages Express VEGF in CNV Membranes

Having established that IL-4 is a potent inducer of sFlt-1 by macrophages, which continues after the removal of IL-4 (Figure 2C) or in the presence of Toll-like receptor ligands and M1-polarizing cytokines (Figure 3), we wanted to test whether this response could be harnessed in the context of neovascular AMD.⁵¹

Initially, a choroidal explant model was used to demonstrate the inhibitory effect of recombinant sFlt-1 on ocular angiogenesis (Supplemental Figure S3) and reinforced the rationale for exploiting the IL-4-mediated response *in vivo*. To test this, we used the experimental model of AMD, L-CNV, which is an archetypal wound-healing, inflammatory-based, and VEGF-mediated model,^{7,52} paralleling pathways operative in AMD in humans. To initially address whether infiltrating macrophages are implicated in VEGF expression, we generated GFP⁺ myeloid chimeric mice through GFP-tg bone marrow transplantation to irradiated mice. Before L-CNV at approximately 6 weeks after transplantation, >85% of peripheral myeloid cells (predominantly monocyte/macrophages) were reconstituted with donor cells (Supplemental Figure S4A), whereas only 0.1% of resident retinal and choroidal CD11b⁺ cells were of donor origin (Supplemental Figure S4A), allowing dissecting the contribution of resident (GFP⁻) versus infiltrating (GFP⁺) cells in the response. After L-CNV, immunohistochemical analysis demonstrated that myeloid-infiltrating GFP⁺ cells, resident host Iba-1⁺GFP⁻ microglia, and pericytes expressed VEGF in the lesion (Supplemental Figure S4B), confirming previous observations.⁷

IL-4 Stimulates sFlt-1 in Recruited and Resident Myeloid Populations *in Vivo*

Macrophages are an important source of VEGF, so modulating macrophage populations toward an antiangiogenic phenotype that secretes sFlt-1 to neutralize VEGF-mediated angiogenesis is a tractable targeted therapy to regulate choroidal neovascularization and to reset immune response thresholds *in vivo*. Initially, we demonstrated that *sFlt-1* expression in the neovascular membranes, arising from the choroid of the eye and infiltrating into the damaged outer structures of the retina (photoreceptors, RPE, and Bruch membrane) at day 3 after laser, is up-regulated *ex vivo* in the presence of exogenous IL-4 (Figure 5A). Intravitreal injection of IL-4 into a normal eye did not detectably increase sFlt-1 mRNA level in whole retinal tissue analysis (Figure 5B), although the macrophage M2

Figure 4 sFlt expression from macrophages is IL-4 dependent. WT or IL-4R^{-/-} BMDMs were stimulated with 20 U/mL IL-4 for 4 hours or 48 hours, and sFlt-1 expression from cells and supernatant fluids was determined by RT-qPCR (A) and ELISA (B) analysis. BMDM, bone marrow-derived macrophage; ELISA, enzyme-linked immunosorbent assay; KO, knockout; nd, not detected; RT-qPCR, quantitative real-time RT-PCR; sFlt-1, soluble fms-like tyrosine kinase 1; WT, wild-type.

Figure 5 IL-4 induces retinal microglia sFlt-1 production. **A:** Gene expression of isolated neovascular membrane after *ex vivo* conditioning with 300 U/mL IL-4 or PBS is presented as fold changes compared with media alone. **B:** RT-qPCR analysis of sFlt-1, Arg-1, Ym1, Vegfa, and Flt-1 expression for whole retina collected at 5 to 48 hours after IL-4 intravitreal administration. **C:** sFlt-1 production was quantified by ELISA on retinal single-cell suspension after 48 hours of culture with IL-4. **D:** Cells were collected after 4 hours of incubation with 300 U/mL IL-4 or PBS, and CD11b⁺ cells were isolated for RT-qPCR analysis. Data are expressed as means $\pm$ SEM. $n = 2$ independent experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus PBS with Mann-Whitney test. ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; RT-qPCR, quantitative real-time RT-PCR; sFlt-1, soluble fms-like tyrosine kinase 1.

marker Ym1 was found to be measurably up-regulated at 24 and 48 hours after injection, showing that retinal cells were activated by IL-4. It was possible to detect significant induction of sFlt-1 protein from whole retinal single-cell suspensions cultured with IL-4 (Figure 5C). This up-regulation was shown to segregate to CD11b⁺ retinal microglia by sorting this cell subset from retina, incubating for 4 hours with IL-4, and analyzing related gene expression (Figure 5D). Microglia showed increased sFlt-1, Arg-1, Ym1, and Flt-1 but not Vegfa mRNA levels. Both full-length and soluble forms of Flt-1 were up-regulated by IL-4, implying IL-4 could enhance Flt-1 promoter activity. RPE was unlikely to be the source of IL-4-induced sFlt-1 and be responsible for CNV inhibition because, first, the increase in IL-4-induced sFlt-1 in primary RPE cells was modest (1.57-fold) (Supplemental Figure S5A) compared with IL-4 stimulation on macrophages (186.7-fold) (Figure 1C), and sFlt-1 mRNA levels remained unaffected (Supplemental Figure S5B). Second, transduction with lenti-mediated shRNA to specifically knockdown sFlt-1 in RPE before CNV induction failed to reverse the IL-4-mediated inhibition of angiogenesis *in vivo* (Supplemental Figure S5C).

Intravitreal Administration of IL-4 Suppresses CNV via Infiltrating Macrophage sFlt-1 Production

Next, we assessed whether IL-4 administration suppressed angiogenesis. Intravitreal IL-4 significantly inhibited CNV development (Figure 6A). To confirm this inhibition was IL-4 specific *in vivo* we performed experiments in IL-4R-deficient animals, wherein significantly larger CNV lesions compared with wild-type animals were observed (Figure 6B).

Because sFlt-1 is up-regulated in CNV membranes after IL-4 administration and because substantial numbers of myeloid cells are associated with CNV evolution,⁷ we tested whether IL-4-induced sFlt-1 from infiltrating myeloid cells was required to inhibit CNV. We developed sFlt-1-knockdown myeloid chimeric mice, generated by transplanting GFP⁺ lineage^{-/-} progenitors transduced by lenti-mediated shRNA to knockdown sFlt-1 (sFlt-1^{KD}). Before L-CNV, >85% of the peripheral CD11b⁺ monocytes were GFP⁺ at 6 weeks after transplantation, whereas only 13% of CD11b⁻CD45⁺ lymphocytes contained lentivirus

Figure 6 IL-4 suppresses laser-induced CNV after intravitreal administration. Choroidal flat mounts after L-CNV were assessed by immune-IB4 staining, and lesions were examined via confocal microscopy from images obtained. CNV size (μm²) of each lesion was quantified with ImageJ software (NIH). **A:** WT eyes treated with IL-4 or PBS via intravitreal route at time of laser were assessed on day 14. **B:** WT versus IL-4R^{-/-} eyes administered with IL-4 assessed on day 7. $*P < 0.05$ versus IL-4R^{-/-} with Mann-Whitney test; $**P < 0.01$ versus no IL-4. CNV, choroidal neovascularization; KO, knockout; L-CNV, laser-induced choroidal neovascularization; PBS, phosphate-buffered saline; WT, wild-type.

Figure 7 IL-4 attenuates L-CNV via induction of sFlt-1 secretion from infiltrating macrophages. Silencing of myeloid-specific-sFlt-1 in bone marrow chimeras after generation and transplantation of transfected lineage-negative precursors. Cells were transduced with GFP lentivirus expressing specific shRNA (K.D.) or scrambled RNA, and FACS isolation of GFP⁺ cells, before transplantation into mice irradiated with a sublethal dose. **A:** At 6 weeks after transplantation, peripheral mononuclear cell populations were processed for FACS analysis. Data are displayed as flow cytometric dot plot representation of successful reconstitutions. **B:** GFP⁺ splenic CD11b⁺ cells underwent FACS, and sFlt-1 expression was analyzed by RT-qPCR. **C:** GFP⁺ bone marrow cells from chimeric animals were isolated and propagated to generate macrophages *in vitro*, to assess their ability to generate sFlt-1 after IL-4 conditioning and signature gene expression via RT-qPCR. **D** and **E:** Quantification of CNV was presented as area and obtained in both bone marrow chimeras and wild-type mice, administered with IL-4 or PBS via intravitreal injection. CNV area was calculated after immunostaining with Col IV and IB4 to delineate margins of the lesion and measured by ImageJ for CNV size (μm^2). Data are expressed as means $\pm$ SEM. $n = 2$ (**A**); $n = 3$ (**B** and **C**); $n = 4$ individual experiments or are representative data of two independent experiments (**D**); $n = 6$ (**E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ with Mann-Whitney test. Scale bar = 50 μ m. CNV, choroidal neovascularization; Col IV, collagen IV; FACS, fluorescence-activated cell sorting; FSC, forward scatter; GFP, green fluorescent protein; K.D., knockdown; L-CNV, laser induced-choroidal neovascularization; PBS, phosphate-buffered saline; RT-qPCR, quantitative real-time RT-PCR; sFlt-1, soluble fms-like tyrosine kinase 1.

(Figure 7A), demonstrating the relative specificity of knockdown to the myeloid subset. Gene silencing efficiency was confirmed by GFP⁺CD11b⁺ splenic macrophages that had impaired sFlt-1 expression compared with scrambled shRNA-transfected control and nonmanipulated animals (Figure 7B). BMDMs developed from GFP⁺sFlt-1^{KD} chimeric animals also failed to express sFlt-1, despite IL-4 conditioning, compared with GFP⁺ scrambled shRNA-transfected control chimeras (Figure 7C). Other macrophage functions were maintained because both control and sFlt-1-silenced macrophages retained equivalent phagocytic capability of RPE debris (Supplemental Figure S6). Intravitreal administration of PBS in shRNA scrambled chimeric animals showed similar CNV development to that seen after laser application to wild-type mice (Figure 7, D and E), indicating that *in vivo* there was no clinical perturbation of

CNV development after lentiviral transduction. Silencing of sFlt-1 in myeloid cells had no effect on their proangiogenic phagocytic behavior, but after intravitreal IL-4 administration the suppression of CNV was attenuated (Figure 7, D and E). Together these experiments demonstrate the mechanism by which IL-4-conditioned infiltrating macrophages suppress angiogenesis in L-CNV and points to a therapy to restore homeostasis and to prevent recurrent new vessel growth.

Discussion

Effective therapeutic regulation of ocular angiogenesis is a critical goal in degenerative and diabetic eye disease. Macrophages express the high-affinity decoy receptor Flt-1^{53,54};

therefore, they possess intrinsic antiangiogenic potential through the production of sFlt-1 by intron retention during the mRNA splicing process. Whether myeloid cells promote angiogenesis or are antiangiogenic is context dependent and disease specific,^{6,55} and here we probe mechanisms by which macrophages can be tuned to deliver antiangiogenic functions.

We have shown that M2 macrophages, conditioned via Th2 cytokines, produce sFlt-1, and limit angiogenesis *in vivo* and *in vitro*. This opens the possibility that Th2 cytokine-mediated sFlt-1 secretion could be therapeutically beneficial after cytokine delivery into the vitreous. Such a positive therapeutic antiangiogenic effect was not secondary to alteration of RPE responses but occurred via myeloid cell conditioning that produced sFlt-1 and inhibited VEGF activity. Such a finding is even more important, because we have recently demonstrated that early infiltrating myeloid cells phagocytose damaged RPE, produce VEGF, and initiate the neovascular process.⁷ Targeting these cells by augmenting CD200R signaling, (resetting macrophage activation threshold) also subverts the angiogenic response.¹¹ Together, these results support the notion that local manipulation of macrophage phenotype and augmenting sFlt-1 secretion could be a powerful strategy to decrease pathologic angiogenesis. We demonstrate in this report that intravitreal injection of IL-4 substantially suppresses L-CNV and, further that, although IL-4 suppression of L-CNV is reversed by inhibiting sFlt-1 secretion from infiltrating macrophages, IL-4 stimulation also has an impact on the function of resident microglia that can produce sFlt-1 and migrate to sites of injury.⁷ Consequently, there may be wider implications for addressing other VEGF-mediated angiogenic disorders such as diabetic retinopathy.

Although IFN- γ induces sFlt-1 in cornea²¹ and LPS is capable of stimulating macrophage-derived sFlt-1 production,²² neither are mechanisms overtly at play during AMD in humans in the absence of an effective T-cell response or Toll-like receptor 4 ligation. So our demonstration of alternative pathways of macrophage-specific sFlt-1 production is novel, circumvents proinflammatory cell activation, and offers a tissue-compliant therapeutic benefit. We examined the extent of sFlt-1 secretion from both M1 and M2 macrophages after conditioning with IFN- γ or LPS and IL-4 or IL-13, respectively. IL-4 or IL-13 (M2), but not LPS or IFN- γ (M1), stimulated macrophages to produce substantial amounts of sFlt-1. Both LPS and IFN- γ suppressed characteristic M2 expression of Arg-1. However, inconsistent with the competitive inhibition of conditions that deliver the M1 or M2 phenotype,⁴⁸ IFN- γ or LPS failed to antagonize promotion of sFlt-1 gene expression by either Th2 cytokine, IL-4 or IL-13, suggesting that IL-4 or IL-13 are, at least under these experimental conditions, dominant sFlt-1 inducers and potentially represent an irreversible default to an antiangiogenic M2 phenotype. This is consistent with studies of CD14⁺ human monocytes that were reported to produce Flt-1 in response to IL-4,⁵⁶ and tumoral

VEGF-macrophage chemotaxis being Flt-1 dependent.⁵³ Taken together, up-regulation of macrophage sFlt-1 and Flt-1 provides a therapeutic strategy by bringing macrophages to a high VEGF-expressing environment (by Flt-1) and then neutralizing proangiogenic signaling in VEGFR2-expressing endothelial cells (by both Flt-1 and sFlt-1).

Arg-1⁺ M2 macrophages are described after conditioning with IL-4, PGE₂, or IL-10.⁵⁷ IL-4 and IL-13 are ascribed to induce M2a macrophages with high expression of Arg-1, mannose receptor 1, and a specific gene signature (Ym1 and *Fizz1*), whereas LPS with additional PGE₂ or IL-1 β stimulates Arg-1[–] and IL-10[–]expressing macrophages with inflammation-regulatory properties (M2b).^{16,58} Combined with our findings that IL-4 antagonizes PGE₂-induced VEGF production (Figure 1), and PGE₂ or IL-10 suppress IL-4[–] or IL-13[–]mediated sFlt-1 production, the data led us to infer that M2a (Ym1⁺*Fizz1*⁺) macrophages are VEGF^{lo}sFlt-1^{hi} and indeed antiangiogenic. It remains of interest to determine whether the Ym1 gene (which critically defines a M2 subphenotype) plays a pivotal role in macrophage regulation of angiogenesis.

In other models, M2 macrophages were shown to promote angiogenesis (eg, wound healing,⁵⁹ tumor development,⁶⁰ and rheumatoid arthritis¹⁵) via VEGF production.⁶¹ Unlike macrophages when activated *in vivo* by proangiogenic growth factors and PGE₂ during wound healing, IL-4[–]conditioned macrophages in our experiments (in the absence of proangiogenic exogenous PGE₂) reflected the effect of IL-4 alone on macrophages that mediate sFlt-1 inhibition of angiogenesis. In addition, PGE₂ is abundant in rheumatoid arthritis synovial liquid⁶² and healing tissue⁶³ and is critical to the induction of IL-10[–]expressing macrophages in solid tumors.⁶⁰ In such PGE₂-rich environments, the effects of IL-4[–]triggered sFlt-1 from macrophages is likely overwhelmed. The balance between IL-4 and PGE₂ dictates whether a phenotype of M2a macrophages or M2b is generated. Measurements of retinal and vitreous PGE₂ levels show minimal elevation, raised by only 70% 1 day after laser injury and resolved 3 days after the induction of CNV.⁵² Therefore, with less PGE₂ production and loss of drive toward M2b VEGF⁺ macrophages in L-CNV, IL-4 administration dominates macrophage conditioning, subverting any PGE₂ effect and M2b generation and inhibiting angiogenesis via M2a[–]sFlt-1 cells.

These results provide evidence that there remain at least two distinct populations of M2 macrophages that either promote (M2b) or antagonize angiogenesis (M2a: IL-4 conditioned Arg-1⁺Ym-1⁺VEGF^{lo}sflt-1⁺); furthermore, we show the therapeutic predominance of IL-4 on regulating macrophage responses (and not through RPE) that attenuates angiogenesis in L-CNV. This approach and the current use of recombinant IL-4 in clinical trials can be exploited therapeutically for ocular neovascular diseases alone or in combination with current anti-VEGF therapies to sustain longer term remission of disease in, for example, nAMD.

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W.-K.W. and D.A.C. designed and performed the experiments, analyzed the data, generated the figures, and wrote the manuscript. A.G., S.L., U.F.O.L., S.J.R., J.L., and J.W. designed and performed experiments; J.W.B. and R.R.A. provided theoretical and technical advice and critical input on the manuscript; D.O.B., L.B.N., and A.D.D. supervised the project, designed the experiments, helped with data interpretation, and wrote the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.04.013>.

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