Persistent Inflammation Subverts Thrombospondin-1–Induced Regulation of Retinal Angiogenesis and Is Driven by CCR2 Ligation

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Neovascular retinal disease is a leading cause of blindness orchestrated by inflammatory responses. Although noninfectious uveoretinitis is mediated by CD4⁺ T cells, in the persistent phase of disease, angiogenic responses are observed, along with degeneration of the retina. Full clinical manifestation relies on myeloid-derived cells, which are phenotypically distinct from, but potentially sharing common effector responses to age-related macular degeneration. To interrogate inflammation-mediated angiogenesis, we investigated experimental autoimmune uveoretinitis, an animal model for human uveitis. After the initial acute phase of severe inflammation, the retina sustains a persistent low-grade inflammation with tissue-infiltrating leukocytes for over 4 months. During this persistent phase, angiogenesis is observed as retinal neovascular membranes that arise from inflamed venules and postcapillary venules, increase in size as the disease progresses, and are associated with infiltrating arginase-1⁺ macrophages. In the absence of thrombospondin-1, retinal neovascular membranes are markedly increased and are associated with arginase-1[−] CD68⁺ macrophages, whereas deletion of the chemokine receptor CCR2 resulted in reduced retinal neovascular membranes in association with a predominant neutrophil infiltrate. CCR2 is important for macrophage recruitment to the retina in experimental autoimmune uveoretinitis and promotes chronicity in the form of a persistent angiogenesis response, which in turn is regulated by constitutive expression of angiogenic inhibitors like thrombospondin-1. This

model offers a new platform to dissect the molecular and cellular pathology of inflammation-induced ocular angiogenesis. (*Am J Pathol* 2012, 180:235–245; DOI: 10.1016/j.ajpath.2011.09.020)

Retinal and choroidal neovascularization are common sight-threatening sequelae that occur in a variety of retinal diseases including age-related macular degeneration (AMD)¹ and various forms of posterior segment intraocular inflammation (PSII).² On the basis of the site of angiogenesis, retinal neovascular membranes (RNM) are classified into subretinal choroidal neovascular membrane (CNV), in which the new blood vessels arise from the choroid and develop most commonly under the retinal pigment epithelium, causing a hemorrhagic detachment, and intraretinal neovascular membrane, or retinal angiomatous proliferation, in which the new vessels grow *de novo* from the retinal vasculature.³ Although only 10% to 20% of AMD patients develop CNV (so-called neovascular AMD or wet AMD),⁴ it accounts for 80% to 90% of AMD-mediated severe visual impairment.^{4–6} In patients with PSII, the incidence of CNV differs in different types of inflammation. In a study by Brown et al, approximately 30% of multifocal choroiditis with panuveitis and 40% of punctate inner choroidopathy developed CNV.⁷ In other studies, 69% to 77% of patients with punctate inner choroidopathy were found to have CNV.^{8,9} CNV is also a common complication of serpiginous choroiditis,^{10,11} posterior sympathetic ophthalmia,^{12–14} angiod streaks,^{15–17} ocular toxoplasmosis,^{18,19} and pathological myopia.^{16,17} Irrespective of spe-

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cific etiology, the common denominator in these disorders is chronic low-grade inflammation or parainflammatory dysregulation of homeostasis that generates activation of innate immunity including complement activation, microglial activation, and macrophage infiltration.²⁰

Although the management of angiogenic diseases has undergone a revolution over the past 5 to 10 years with the use of anti-vascular endothelial growth factor (VEGF) antibody,²¹ RNM remains a major cause of blindness in the working population.² Perhaps not surprisingly, because VEGF is a fundamental regulator of angiogenesis responsible for pathological blood vessel growth and increased microvascular permeability, VEGF blockade has been relatively successful in the clinics. However, neutralizing VEGF activity per se does not remove the underlying drive for angiogenesis, which includes inflammation. Chronic inflammation and angiogenesis are coupled together not only in the aforementioned retinal conditions, but also in many other disorders, including rheumatoid arthritis, psoriasis, Crohn's disease, diabetes, and cancer. In rheumatoid arthritis, new blood vessels are often formed at the early stages of inflammation, resulting in increased leukocyte ingress into the synovium,²² where leukocyte-endothelial interaction may play an important role in the initiation and progression of synovial angiogenesis,²³ although during cancerogenesis, the ischemic microenvironment and tumor-associated macrophages are central for tumor angiogenesis.²⁴ In the neuroretina, however, detailed mechanisms on how angiogenesis is formed (either from the choroid or *de novo* from the retina) in the presence of chronic inflammation remain poorly defined.

Posterior segment intraocular inflammation is modeled in rodents by immunizing with retinal specific antigens including S antigen and interphotoreceptor retinoid binding protein (IRBP) (experimental autoimmune uveoretinitis, EAU).^{25–28} The model system has greatly advanced our understanding of the mechanism of disease development and has provided an excellent preclinical model for testing therapeutic agents for human PSII.^{28–31} In EAU, although it is evident that disease clinically remits, a low-grade inflammatory retinal infiltrate (principally mononuclear cells) persists, and there is no evidence that homeostatic reconstitution in terms of cell numbers or activation status^{32,33} occurs up to 60 postimmunization (p.i.) days. In the present study, we provide further evidence that, after an initial acute episode, retinal inflammation persists for over 4 months and is associated with a pathological angiogenic response, ie, RNM. RNMs during EAU are initially formed from inflamed retinal venules or postcapillary venules in the inner retina, then migrate or become relocated toward the outer layers as these cellular layers degenerate, ultimately invading the retinal pigment epithelial (RPE) layer and forming chorioretinal anastomoses with focal RPE cell detachment. Although this model system mimics the development of CNV in PSII patients, the dynamic migration of neovascular membrane from the neuroretina to the RPE layer also imitates the clinical setting of the progression of retinal angioma-tous proliferation.³ We further show that the angiogenic process is more aggressive in the absence of thrombospondin-1 (TSP-1) while it is reduced when CCR2–CCL2

(chemokine C-C motif ligand 2) ligation is disrupted and macrophages are relatively absent from inflamed tissue, indicating a direct regulation of angiogenesis by TSP-1 and a partial requirement for CCR2⁺ myeloid cells for the angiogenic response.

Materials and Methods

Animals

Eight- to 12-week-old wild-type C57BL/6 and CCR2-deficient mice (B6.126S4-Ccr2^{tm1lfc}/J, originally purchased from Jackson Laboratory, Bar Harbor, ME)³⁴ were bred and maintained in the medical research facility of the University of Aberdeen. Thrombospondin-1-deficient (TSP-1^{–/–}) mice (C57BL/6 background) were originally from Jack Lawler³⁵ and were maintained in the biomedical resource facility of the University of Bristol at BC10 (back-crossed 10 generations). All animals were housed under conditions outlined in the Home Office Code of Practice and according to the Home Office Regulations for Animal Experimentation (UK) and with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

EAU Induction and Clinical Evaluation

EAU was induced using a previously described protocol.^{34,36,37} Briefly, the animals were immunized with 500 μ g IRBP peptide 1–20³⁸ (GPTHLFQPSLVLDMAKVLLD; Sigma-Genosys, Cambridge, UK), emulsified 1:1 in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) with the addition of 2.5 mg/mL *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories). An additional 1 μ g of *Bordetella pertussis* toxin (Health Protection Agency, Salisbury, UK) was administered intraperitoneally at the time of IRBP immunization. At different days p.i., retinal inflammation was accessed clinically using the topical endoscopic fundus image^{33,39} and scored using criteria described previously.³⁹ For fluorescein angiography, mice were injected via the tail vein with 100 μ L of 2% (w/v) sodium fluorescein (Sigma-Aldrich, St. Louis, MO), and images were recorded digitally using a home-built scanning laser ophthalmoscope.⁴⁰

Histopathology

Mouse eyes were collected on days 25, 80, and 120 p.i. ($n = 6$ mice at each time point) and fixed in 2.5% (w/v) glutaraldehyde (Fisher Chemicals, Loughborough, UK) and embedded in paraffin for standard H&E staining. The structural and infiltrating scores of retinal inflammation were graded by two independent observers using a scoring system described previously.⁴¹

Confocal Microscopy of Retinal Flat Mounts

Eyes were collected at days 25, 60, 80, and 120 p.i. ($n = 6$ to 8 mice at each time point), then fixed in 2% paraformaldehyde (Fisher Chemicals). Flat-mount

preparation and staining were performed as previously described.^{42,43} Briefly, retinas were dissected and then permeabilized in 0.3% Triton X-100 in PBS, followed by 5% bovine serum albumin blocking solution. Samples were then incubated with primary antibodies at 4°C overnight. After being thoroughly washed, samples were incubated with second antibodies for 3 hours at room temperature in the dark. After final-step incubation, samples were washed in PBS and mounted flat on glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were examined with a META LSM510 confocal microscope (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). LSM Image Browser software was used for image analysis (Carl Zeiss MicroImaging GmbH).

All confocal images were analyzed by the LSM Image Browser software version 4.2 (Carl Zeiss MicroImaging GmbH). The size (area) of neovascular membrane was measured in merged images from a series of Z-stack images. The numbers of neovascular membrane were counted manually in low-magnification confocal images (with 5× objective lens).

Immunohistochemistry

Mouse eyes were enucleated and embedded in optimal cutting temperature compound (Thermo Fisher, Loughborough, UK), and stored at -80°C until use. Serial sections (6 µm thick) were cut with a cryostat and fixed in 2% paraformaldehyde for 10 minutes. Sections were washed and then blocked with 5% bovine serum albumin for 30 minutes. Samples were incubated with rabbit anti-mouse collagen IV (1:100, AbD Serotec) and goat anti-CCL2 (1:100; R&D Systems, Minneapolis, MN) or rat anti-mouse VEGF (1:100; Abcam Plc., Cambridge, UK) for 1 hour, followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG and Alexa Fluor 546 conjugated donkey anti-goat IgG (Invitrogen, Paisley, UK), or phycoerythrin-conjugated anti rat IgG (1:100; BD Biosciences, Oxford, UK) for a further hour. After final washing, sections were mounted with Vectashield Mounting Media (Vector Laboratories) and examined with the META LSM510 confocal microscope as above.

Cytokine Measurement

Supernatants of splenocytes from day 25 and day 120 p.i. EAU mice ($n = 6$) cultured with or without 25 µg/mL of IRBP peptide 1–20 were analyzed for tumor necrosis factor- α , interferon (IFN)- γ , IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, and IL-21 using the fluorescence-activated cell sorting cytometric bead assay kit (BD Biosciences).

RNA Isolation and Reverse Transcription and Real-Time RT-PCR

Retinas were dissected and snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted using the RNeasy mini kit (Qiagen Ltd., West Sussex, UK) following the manufacture's instruction. The quantity and quality of the RNA were determined by

NanoDrop ND-1000 spectrophotometer (Thermo Fisher). cDNA were reverse transcribed with the same amount of total RNA using the SuperScript II Reverse Transcriptase kit and random primer (Invitrogen).

Real-time RT-PCR for *Tgfb*, *Alk1*, *Alk5*, and *Thbs1* was performed using the LightCycler 480 system (Roche Applied Science, Mannheim, Germany) in 384-well plates using the LightCycler 480 SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany). Primers were designed using the Web tool "primer 3" and synthesized by Sigma-Aldrich. Primer sequences are as follows: *Alk1*: forward 5'-GAAGCAGCGGGATTGAC-3'; reverse 5'-TACCGTCTCTGCACCAAGAA-3'; *Alk5*: forward 5'-TCCAACTACAGGACCTTTTCA-3'; reverse: 5'-GCAGTG-GTAAACCTGATCCAGA-3'; *Tgfb*: forward: 5'-GTGTGG-AGCAACATGTGGAAC-3'; reverse: 5'-GGGCTGATC-CCGTTGATTTC-3'; *Thbs1*: forward: 5'-TGTGGACTTC-AGCGGTACCTTCTT-3'; reverse 5'-GGACTGGGTGACT-TGTTTCCACAT-3'; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): forward: 5'-ACTTTGTCAAG-CTCATTTC-3'; forward: 5'-ACTTTGTCAAGCTCATT-TCC-3'. The expression of *Ccl2* and *Vegf* genes were analyzed by TaqMan gene expression assay (*Vegf*: Mm01281449_m1. *Ccl2*: Mm9999056_m1). Absolute quantification and the SD method were used for data analysis. Gene expression level was normalized to the housekeeping gene *GAPDH*.

Data and Statistical Analyses

Real-time RT-PCR data were analyzed by one way analysis of variance multiple comparison Dunnett's test, and cytokine production by splenocytes from different days of EAU mice were compared by Student's *t*-test. EAU clinical and histological scores were compared by the Mann-Whitney test. All data were presented as mean $\pm$ SEM. $P < 0.05$ were considered statistically significant.

Results

Retinal Inflammation during Different Stages of EAU

In C57BL/6 mice, retinal inflammation developed at 10 to 14 days p.i. and peaked at 21 to 28 days p.i. (Figure 1, B and I). As the disease progressed, the severity of retinal inflammation declined, but chronic inflammation persisted for more than 120 days (Figure 1I). During the acute phase (ie, days 10 to 28 p.i.), the inflammation was characterized by retinal edema, particularly around the optic disk; engorgement and sheathing of venules; and multiple infiltrates (Figure 1B).³⁹ In the chronic stages of EAU, the fundus appeared gray or pale in color (Figure 1, C–H) and often displayed both active lesions and old scars (Figure 1, E and G). Active lesions included vasculitis (Figure 1, C and D) as well as multiple infiltrates in the retina (Figure 1F) and in the inferior vitreous gel (Figure 1D). Vascular sheathing/cuffing during the chronic stages of EAU appeared as relatively clear, pale sharp edges surrounding the vessels (Figure 1, C and F), com-

Figure 1. Clinical and histological presentations of retinal inflammation at different stages of EAU. Mice were immunized with IRBP 1–20 peptide, clinical and histological investigations (*Materials and Methods*) performed at different p.i. days. **A:** Normal nonimmunized mouse retina. OD, optic disk. **B:** Day 25 p.i. mouse fundus showing severe optic disk inflammation, vasculitis (**white arrows**). **C:** Day 60 p.i. mouse fundus showing retinal atrophy, small retinal infiltrates, and vascular sheathings (**white arrows**). **D:** Day 80 p.i. mouse fundus showing vascular sheathing and inferior vitreous infiltrates (**asterisks**). **E:** Day 80 p.i. mouse fundus showing multiple infiltrates and an area of gliosis or scar (**blue arrow**). **F:** Day 90 p.i. mouse fundus showing multiple retinal infiltrates (**arrowheads**) and vascular sheathing (**arrow**). **G:** Day 120 p.i. mouse fundus showing large scars (**blue arrows**). **H:** Day 120 p.i. mouse fundus showing pigment depositions. **I:** Clinical score of retinal inflammation. **J:** H&E staining of a retinal section from a normal mouse. **K:** Retinal section from a day 25 p.i. mouse showing massive inflammatory cell infiltration and retinal folds (**arrows**). **L:** Retinal section from a day 80 p.i. mouse showing structural damage and retinal scars (**arrowheads**). **M:** Retinal section from a day 120 p.i. mouse showing structural damage and a big scar extending from inner retina to outer retina (**arrowheads**). **N:** Retinal section from a day 120 p.i. mouse showing ghost vessel (**asterisk**) connecting with a scar (**arrowhead**). **O:** Section from a day 120 p.i. mouse showing a neovascular complex (**black arrow**) connecting with RPE cells. **P:** Histological scores (cell infiltration and structure damage) of retinal inflammation at different p.i. days. GL, ganglion layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelia; Ch, choroid.

pared to the blurred margins seen during the acute phase (Figure 1B), and the affected vessels often clinically appeared underperfused and consequently of reduced caliber and whitish in color (Figure 1B with Figure 1F). Similarly, retinal infiltrates in the chronic phases had more distinct borders, were atrophic in appearance, and were often associated with retinal vessels (Figure 1, D and F). Occasionally, pigment deposits on the retinal surface were observed in the late stages (80 to 120 days p.i.) (Figure 1H).

Fluorescein angiography of the fundus revealed an intact blood–retinal barrier in normal nonimmunized mice (see Supplemental Figure S1A at <http://ajp.amjpathol.org>), whereas in day 25 p.i. mice, substantial diffuse fluorescein leakage was observed in the venules and postcapillary venules, resulting in blurring of the vessel wall margins and overall obscuration of the fundal view (see Supplemental Figure S1B at <http://ajp.amjpathol.org>). By day 60 p.i. onward, the view of the fundus had improved, and fluorescein leakage was restricted to discrete segments of postcapillary venules (see Supplemental Figure S1, C–E, at <http://ajp.amjpathol.org>). The size of the hyperfluorescent lesions appeared to increase

from day 60 to 120 p.i. (see Supplemental Figure S1, C–E, at <http://ajp.amjpathol.org>).

Histological examination showed that in day 25 p.i. mice (Figure 1K), retinal thickness increased massively compared to nonimmunized controls (Figure 1J), and infiltrating inflammatory cells were observed throughout the layers of the posterior segment of the eye (Figure 1K). Additionally, structural changes were common, with retinal folds frequently observed (Figure 1K), as well as disruption of the retinal architecture (Figure 1, J and K). As the disease progressed toward the chronic stages, the number of infiltrating cells declined and the thickness of the retina reduced, whereupon retinal structure was partially restored (Figure 1, L and M). However, in the areas where photoreceptors were destroyed, fibrotic changes (Figure 1, L and M) were observed and retinal structure remained disordered. In addition, extensive perivascular leukocyte accumulation was observed during chronic EAU (Figure 1N). Occasionally, a ghost vessel was observed connected to a patch of gliosis, which extended from the ganglion cell layer to the outer retinal layers (Figure 1N). Interestingly, blood vessels were frequently detected at the outer layer of the retina (Figure

Figure 2. Cytokine production by spleen cells of day 25 and 120 p.i. mice. Splenocytes were isolated from day 25 and day 120 p.i. mice and cultured in the absence or presence of 25 μ g/mL IRBP peptide 1–20 for 72 hours. Cytokine production in the supernatants was measured with the cytometric bead assay kit. **A:** Cytokine production by cells from day 25, and day 120 p.i. mice in the absence of IRBP stimulation. **B:** Cytokine production by cells from day 25 p.i. mice. **C:** Cytokine production by cells from day 120 p.i. mice. * $P < 0.05$, ** $P < 0.01$ compared to IRBP (–) group. Student's t -test, $n = 6$.

10), and they were often connected to RPE cells (Figure 1O). The structural and infiltration score of EAU at day 80 and 120 p.i. mice was significantly lower than that at day 25 p.i. mice (Figure 1P), but importantly emphasized the persistence of an inflammatory response.

Cytokine Production at Different Stages of EAU

In the absence of recall responses to IRBP peptide, spleen cells from day 25 p.i. EAU mice produced small amounts of inflammatory cytokines including tumor necrosis factor- α , IL-6, IL-17, IL-21, IL-12, and anti-inflammatory cytokine IL-10 (Figure 2A), whereas the production of these cytokines was negligible by cells from day 120 p.i. EAU mice (Figure 2A). When the cells were reactivated with IRBP peptide *in vitro*, the production of IFN- γ , IL-2, tumor necrosis factor- α , IL-6, and IL-17 was significantly increased by cells from day 25 p.i. EAU mice (Figure 2B). Cells from day 120 p.i. EAU mice also produced markedly elevated inflammatory cytokine levels after IRBP peptide stimulation *in vitro* (Figure 2C). However, the level of IFN- γ production was significantly less than that of day 25 p.i. mice ($P < 0.05$; Figure 2, B and C).

Retinal Angiogenesis at Late Stages of EAU

Confocal microscopy of retinal flat mounts using antibody to type IV collagen staining to identify vascular elements revealed no abnormal new blood vessels in day 25 p.i. EAU retina (Figure 3A). In contrast, retinas from day 60 p.i. mice showed small patches of new blood vessels (Figure 3B), and by day 80 p.i., these new vessels had formed membranous sheets (Figure 3C). New blood vessels arose directly from retinal venules (Figure 3D), and postcapillary venules formed a membrane (RNM) (Figure 3E), which was morphologically different from the surrounding normal retinal vasculature.

Z-stack scanning confocal imaging of retinal flat mounts showed that the RNM extended from the inner retina to the outer retina, where they fused with RPE cells (Figure 3F). Interestingly, patches of RPE cells were consistently associated with the RNM (Figure 3, F, G, I–K), which allowed their ready identification. The number of RNM peaked at approximately day 80 p.i. (Figure 3H); however, the size of neovascular membranes continued to increase from day 80 to day 120 p.i. (Figure 3, I–L).

Because macrophages play a key role in angiogenesis, we examined the RNM complexes for macrophage infiltration. In the inflamed retinas, F4/80 $^{+}$ macrophages and CD4 $^{+}$ T cells were detected in large numbers in day 25 p.i. mice (Figure 3M). However, in day 80 p.i. mice, macrophages were detected predominantly surrounding and within areas of neovascular membranes (Figure 3N). Dual staining of retinal flat mounts for CD68 and arginase-1 revealed CD68 or arginase-1 single positive cells, but no dual-positive cells (Figure 3, O–Q). From day 25 to day 120 p.i., the proportion of CD68 $^{+}$ cells declined, whereas the proportion of arginase-1 $^{+}$ cells increased (Figure 3, O–R).

Growth Factors and Chemokine CCL2 Expression in the Inflamed Retina

Preferential accumulation of inflammatory cells in the areas of neovascular membrane strongly suggests a causal role for these cells in retinal angiogenesis in this setting. To further understand the molecular mechanism, we investigated gene expression of transforming growth factor (TGF)- β , TGF- β receptors, TSP-1, VEGF,⁴⁴ and chemokine CCL2 that are likely to be produced by infiltrating macrophages and are known to be crucial in pathogenic retinal angiogenesis.⁴⁵ The expression of TGF- β 1 mRNA increased over 12-fold in the retinas from day 80 p.i. EAU mice as compared to that from nonimmunized control mice (Figure 4A). Interestingly, the expression of type 1 TGF- β receptor ALK1 (Figure 4C), which initiates the angiogenic signaling pathway⁴⁶ but not the ALK5 (Figure 4B), which initiates the antiangiogenic pathway,⁴⁶ was upregulated in day 80 p.i. mice. TSP-1 is a critical enzyme for conversion of the latent form of TGF- β into active form. The mRNA expression of Thbs1 gene did not change in the retina of day 25 p.i. mice but significantly increased in day 80 p.i. mice (Figure 4D). There was no significant increase in expression of vegf mRNA in day 25 p.i. mouse retinas (Figure 4E); however, vegf mRNA expression increased 2.7-fold in day 80 p.i. mouse retinas as compared to nonimmunized controls (Figure 4E). In addition, immunohistochemistry detected negligible levels of VEGF protein in the retina from normal nonimmunized (Figure 4F) and day 25 p.i. mice (Figure 4G), but a significant level of VEGF expression was detected in the areas of neovascular membrane in day 80 p.i. mice, particularly within the vessels (Figure 4H).

Figure 3. Confocal microscopy of retinal flat mounts at different stages of EAU. Flat-mount retinas were stained for collagen IV (A–F, I–K), or F4/80 and CD4 (M and N) and collagen IV (N), or CD68 and arginase-1 (O–Q) and observed by confocal microscopy. **A:** Image from day 25 p.i. mouse showing normal retinal vasculature. **B:** Image from day 60 p.i. mouse showing new blood vessels arising from retinal venules (arrows). **C:** Image from day 80 p.i. mouse showing neovascular complex (arrows). **D:** High-magnification image from day 60 p.i. mouse showing new vessels arising from retinal venules. **E:** Neovascular complex connecting with a postcapillary venule. **F:** Z-stack images from day 80 p.i. mouse showing neovascular complex (large arrow) extending from inner retina to outer retina (small arrows) causing RPE detachment (black area). **G:** Phase contrast image of retinal flat mount from a day 80 p.i. mouse showing patches of detached RPE cells in the areas of neovascular complex. **H:** Number of retinal neovascular complex in mice of different days of EAU. **I–K:** Representative images from day 60 (I), day 80 (J), and day 120 (K) p.i. mice showing the size of neovascular membrane. **L:** Average size of retinal neovascular membrane in mice of different days of EAU. * $P < 0.05$, ** $P < 0.01$ compared to day 60 p.i. mice, Dunnett's test; $n \geq 12$, no neovascular complex was detected. **M:** Image from day 25 p.i. mouse retina stained for F4/80 (red) and CD4 (green). **N:** Image from day 80 p.i. mouse retina stained for F4/80 (red), CD4 (blue), and collagen IV (green). **O–Q:** Images from day 25 p.i. (O), day 60 p.i. (P), and day 120 p.i. (Q) mouse retinas stained for CD68 (red) and arginase-1 (green). **R:** Relative proportion of CD68⁺ cells and arginase-1⁺ cells in the retinas in different stages of EAU.

The expression of *ccl2* mRNA in the retina increased 30-fold in day 25 p.i. mice and 76-fold in day 80 p.i. mice compared to nonimmunized control mice (Figure 4I). CCL2 protein was not detected in normal nonimmunized mouse retina (Figure 4J) but was present at significant levels in day 25 p.i. mouse retina (Figure 4K), and the expression was further increased and localized mainly around the neovascular membrane areas in day 80 p.i. mouse retinas (Figure 4L).

Deletion of TSP-1 Enhances Chronic Inflammation-Mediated Retinal Angiogenesis

Increased ALK1 but not ALK5 expression in chronic EAU suggests that TGF- β may play a role in the angiogenic response in this setting. To further examine the role of TSP-1/TGF- β 1 in retinal angiogenesis in this model, EAU was induced in TSP-1^{-/-} mice. A previous study has shown that deletion of the *Thbs1* gene resulted in a more intense acute EAU.⁴⁷ At day 80 p.i., wild-type (WT) (Figure 5A) and TSP-1^{-/-} (Figure 5B) mice manifested similar levels of retinal inflammation (Figure 5C). Interestingly, retinal neovascular membrane was particularly enhanced in TSP-1^{-/-} mice (Figure 5E) compared to WT mice (Figure 5, D and F). Unlike the WT mice, where many arginase-1⁺ cells were detected during late stages of EAU

(Figures 3R and 5G), only few arginase-1⁺ cells were detected in the retinas from day 80 p.i. TSP-1^{-/-} mice (Figure 5, H and I). Bone marrow–derived macrophages from TSP-1^{-/-} mice, however, demonstrated equal ability as WT bone marrow–derived macrophages to respond to either M1 or M2 conditioning, and to express nitric oxide (NO), upregulate nitric oxide synthase 2 (*NOS2*) or *Arg1* or the *Tgfb* gene, and generate VEGF (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

Deletion of CCR2 Reduces Chronic Inflammation-Mediated Retinal Angiogenesis

The CCL2–CCR2 pathway is known to be important for macrophage and hematopoietic stem cell recruitment to sites of inflammation.⁴⁸ A previous study has shown that this pathway is involved in angiogenesis,⁴⁵ and currently we have enumerated high levels and localized expression of CCL2 during the chronic phase of EAU. Previous studies have shown that deletion of CCR2 does not affect the expression of EAU^{49,50} but may modify the nature of the inflammatory cell infiltrate (eg, neutrophils, but not macrophages, dominate the inflamed retina^{49,50}). In this study, at day 80 p.i., we observed no significant difference in EAU inflammatory activity between WT and CCR2 knockout mice (Figure 6A). Histologically, fibrotic scar-

ring was frequently observed in the retina of WT mice (Figure 6, B and C). Such changes were less frequent in CCR2 knockout mice (Figure 6, D and E). Retinal flat-mount investigation ($n = 6$) demonstrated significantly less retinal neovascular membrane in CCR2-deficient mice as compared to WT mice (Figure 6, F–H). In addition, the size of retinal neovascular membrane in CCR2 knockout mice was significantly smaller than those observed in WT mice (Figure 6, I–K). Dual staining of day 80 p.i. mouse retina for major histocompatibility complex-II and *Gr-1* (granulocyte differentiation antigen-1) revealed many *Gr-1*⁺ cells in CCR2 knockout mice (Figure 6M). WT mouse retina was filled with major histocompatibility complex-II⁺ *Gr-1*⁺ cells (Figure 6L).

Discussion

Here, we showed that retinal inflammation from EAU in C57BL/6 does not spontaneously resolve. After the initial acute inflammation (10 to 28 days p.i.), chronic disease persists for over 4 months. Of more significance is that we show that chronic inflammation in EAU induces retinal angiogenesis. Unlike other physiological (female reproductive system) or pathological conditions (carcinogenesis, wound healing) of vessel growth, new blood vessels in chronic EAU arise from the inflamed venules and post-capillary venules in the inner retina forming vascularized

membranes, which morphologically differ from normal vasculature. Once formed in the inner retina, the neovascular membranes then migrate toward the outer retina, causing RPE detachment. The model therefore mirrors inflammatory CNV observed in patients with long-standing chronic choroiditis² and provides a unique opportunity to dissect the role of inflammation as a cause of angiogenesis and the mechanism underlying how inflammation drives—or indeed may control—pathological ocular angiogenesis.

EAU is a CD4⁺ T-cell-mediated autoimmune disease mediated by both Th1 and Th17 cells.^{51,52} What causes the inflammation to persist is not known. In the C57BL/6 model, inflammation is initiated by IRBP peptide 1–20-specific CD4⁺ T cells and IRBP antigen in photoreceptors remains the primary target. Although many photoreceptors are damaged during the acute phase of EAU, areas where normal, healthy photoreceptors remain detectable in the chronic stages in this mouse model (Figure 1). The remaining IRBP (auto)antigen in undamaged photoreceptors is likely to be one of the continued drivers for persistence of inflammation, along with antigen and epitope spreading. Indeed, spleen antigen-specific memory T-cell responses can be elicited late in the disease (Figure 2), although interestingly, this has shifted more toward a Th17 rather than a Th1 response.

Figure 4. Expression of growth factors in mouse retina during different stages of EAU. The expression levels of mRNA in mouse retinas were determined by real-time RT-PCR, and the protein levels were examined by confocal microscopy. **A:** TGF-β1 mRNA expression. **B:** ALK5 mRNA expression. **C:** ALK1 mRNA expression. **D:** TSP mRNA expression. **E:** VEGF mRNA expression. VEGF (red) and collagen IV (green) expression in the retina of normal (**F**), day 25 p.i. (**G**), and day 80 p.i. (**H**) mouse. **I:** CCL2 mRNA expression. CCL2 protein (red) and collagen IV (green) expression in normal (**J**), day 25 p.i. (**K**), and day 80 p.i. (**L**) mouse. ONL, outer nuclear layer; RPE, retinal pigment epithelia; Ch, choroid. **Arrows**, retinal neovascular complex. * $P < 0.05$, ** $P < 0.01$, Student's *t*-test (**A–D**) and Dunnett's multiple comparison test (**E** and **I**); $n = 6$ to 8.

Figure 5. Retinal neovascular membrane in TSP^{-/-} mice. EAU was induced in WT and TSP^{-/-} mice. Clinical inflammation was examined by topical endoscopic fundus imaging. At day 80 p.i., mice were sacrificed and retinal flat mounts were stained for collagen IV and observed by confocal microscopy. Retinal inflammation in a day 80 p.i. WT (A) and a TSP^{-/-} mouse (B). C: Clinical score of retinal inflammation at day 80 p.i. WT (D) and TSP^{-/-} mouse (E). F: Histogram showing the size of retinal neovascular membrane in WT and TSP^{-/-} mice. **P* < 0.05, unpaired Student's *t*-test, *n* = 6. Confocal images of retinal flat mounts showing CD68⁺ (red) and arginase-1⁺ (green) cells in a day 80 p.i. WT (G) and a day 80 p.i. TSP^{-/-} (H) mouse. I: Histogram graph showing the relative proportion of CD68⁺ and arginase-1⁺ cells in day 80 p.i. WT and TSP^{-/-} mice (*n* = 4).

The macrophage infiltration associated with the late angiogenic response also shows interesting phenotypic change. In the acute stage of the disease (day 25 p.i.), infiltrating macrophages were CD68⁺ arginase-1⁻, whereas in the later stages, there was a substantial in-

crease in Arg-1⁺ macrophages. In other studies, it has been shown that extent of autoimmune disease is therefore limited in later stages by the generation of regulatory networks, including regulatory T cells and myeloid-derived suppressor cells.³² In the current model of EAU, regulatory mechanisms appear only to have partial effect because although the acute phase subsides, the disease persists as a chronic inflammation, is associated with a predominance of Th17 T cells, and presents with an arginase-1⁺ macrophage-infiltrated foci of neovascularization and fibrosis in the form of neovascular membranes. Persistence of Th17 cells over Th1 cells in chronic inflammatory conditions has been observed in a number of settings, including tuberculosis,⁵³ arthritis^{54,55} and colitis.⁵⁶ Indeed, IL-17 is linked to inhibition of apoptosis which would promote resolution, and so this cytokine helps maintain inflammation.^{54,55}

Although EAU in the late stages persists as a chronic disease, the fibrovascular "healing" response in the retina may represent an attempt at regulation of inflammation and retinal damage. Photoreceptors, blood vessels, and other retinal cells/molecules are damaged either directly by antigen-specific T cells or indirectly by inflammatory mediators during the acute phase of EAU. The increase in highly phagocytic arginase-1⁺ macrophages (putative alternatively activated M2 macrophages⁵⁷) is consistent with a shift in outcome from tissue damage to tissue repair and removal of damaged or dead cells/molecules during the wound healing process.⁵⁸ Arginase-1 is involved in proline and polyamine biosynthesis; the former promote extracellular matrix membrane construction, whereas the latter are involved in cell proliferation.⁵⁹ In addition, retinal tissues from day 80 p.i. EAU mice also expressed high levels of TGF-β1 and VEGF (Figure 4), further corroborating a drive toward healing rather than destructive inflammation.

Specific molecular mechanisms almost certainly direct retinal inflammation in late-stage EAU toward a

Figure 6. Retinal neovascular membrane in CCR2-deficient mice. EAU was induced in WT and CCR2 knockout mice. At day 80 p.i., mice were sacrificed and retinal flat mounts were prepared for confocal microscopy. A: Clinical score at day 80 p.i. B and C: Histology of EAU retina from day 80 p.i. WT mice. D and E: Histology of EAU retina from day 80 p.i. CCR2^{-/-} mice. Phase contrast images of retinal flat mounts from a WT mouse (F) and a CCR2 knockout mouse (G). Numbered pigmented areas represent retinal neovascular membrane with RPE detachment. H: Average number of retinal neovascular membrane in WT and CCR2 knockout mice at day 80 p.i. ***P* < 0.01, unpaired Student's *t*-test; *n* = 12 eyes. Representative confocal images showing the size of neovascular membrane in WT (I) and CCR2 knockout (J) mice. K: Average size of neovascular membrane in WT and CCR2 knockout mice. ***P* < 0.01, unpaired Student's *t*-test, *n* = 12 eyes. Confocal images showing major histocompatibility complex-II⁺ (green) and Gr-1⁺ (red) cells in day 80 p.i. WT (L) and CCR2 knockout (M) mice.

wound healing response. Angiogenesis is an intrinsic component of the wound healing process, and macrophages are central to this process. Here we have shown that the late-stage EAU retinal angiogenic response and the macrophage infiltration are significantly reduced in the absence of CCR2–CCL2 ligation. CCR2 is expressed on inflammatory macrophages and is a critical receptor involved in recruitment of macrophages to sites of inflammation,^{60,61} including the retina in EAU.³⁷ Clearly, CCR2 is involved in the full expression of late-stage EAU retinal angiogenesis. Macrophage-induced angiogenesis has the potential to be fine-tuned. While “inflammatory” macrophages (M1) are robust producers of inflammatory cytokines involved in tissue damage, including VEGF, and so have angiogenic potential,^{62,63} “regulatory” macrophages (M2) have significantly less proinflammatory effect while still having the capacity to produce VEGF. The balance between M1 and M2 macrophages is thus shifted in late EAU toward, but not solely, angiogenesis. In CCR2-deficient mice, where macrophage numbers are sparse and the infiltrate is predominantly neutrophilic (Figure 6), the angiogenic response is concomitantly reduced.

Healing responses, however beneficial they are to the survival of the host, may be damaging to delicate ocular tissues. Scar tissue in particular is a major threat to ocular function.⁶⁴ The eye, and especially the retina, secretes many immunomodulatory molecules, which contribute to immunological privilege⁶⁵ and include growth factors such as TGF- β ^{66,67} and TSP-1. TSP-1 has been shown to be important in regulating the severity of the acute response in EAU⁴⁷ and is produced constitutively by retinal cells such as RPE, presumably to mediate this regulatory role. We show here that TSP-1 is also important in regulating the retinal angiogenic response because in its absence, persistent inflammation mediated retinal angiogenesis is markedly increased (Figure 5). It would thus appear that whereas inflammatory and, later, proangiogenic macrophages are necessary for the full expression of EAU and in particular for its progression to chronicity, TSP-1, in concert with many other local regulatory factors, attempts to exert control over the damaging effects of inflammation and modulate its outcome. In the absence of TSP-1, although macrophages are able to respond to M1 (lipopolysaccharide and IFN- γ) and M2 (IL-4, IL-13, and prostaglandin E2) conditioning and differentiate into NOS2, or arginase-1-expressing cells *in vitro* (see Supplemental Figure S2 at <http://ajp.amjpathol.org>), in the inflamed retina of late-stage EAU, macrophage infiltrates have remained inflammatory phenotype (CD68⁺arginase-1[−] M1 type macrophage) and are angiogenic.^{62,63} The overall outcome, therefore, is increased angiogenesis as a result of the lack of constitutive retinal TSP-1.

In summary, we have described here a new model of chronic inflammation in the retina in the C57BL6 mouse. The model is characterized by persistent infiltration of arginase-1⁺ CD68 macrophages and discrete sheet-like foci of membranous neovascular complexes. This retina-specific angiogenic response requires CCR2–CCL2 ligations to promote macro-

phage recruitment to the lesions and is regulated by tissue expression of the angiogenic inhibitor TSP-1. This model closely resembles the inflammation-induced angiogenesis that occurs in human posterior segment intraocular inflammation, and investigation of its molecular mechanisms will provide insight into the pathogenesis of ocular angiogenesis generally.

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