Potential Role of Microglia in Retinal Blood Vessel Formation

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Purpose. The role of microglia, present in the retina early in development before vascularization, remains ill defined. The authors investigated whether microglia are implicated in retinal blood vessel formation.

Methods. The microglia and vasculature of developing human fetal and rodent retinas were examined by labeling the endothelial cells with lectin and the microglia with CD18 antibody or green fluorescent protein driven by the promoter of the chemokine receptor CX_3CR1. Rodent ischemic proliferative retinopathy induced by hyperoxia or hypercapnia, which model retinopathy of prematurity, and ex vivo retinal explants were used to assess microglial involvement in vascular pathology. Microglial participation in developmental retinal vessel formation was further studied in neonatal rats after pharmacologic macrophage depletion with the use of clodronate liposomes and subsequent intravitreal injection of microglia.

Results. Microglia intimately appose developing vessels of human and murine retinas. Ischemic retinopathy models exhibit decreased microglia concomitant with the characteristic reductions in vasculature observed in these retinopathies. Retinal explants exposed to conditions resulting in ischemic retinopathies (in vivo) reveal that antioxidants protect against microglial loss. Depletion of resident retinal microglia, but not systemic macrophages, reduced developmental vessel growth and density, which were restored by intravitreal microglial injection.

Conclusions. These observations suggest that proper retinal blood vessel formation requires an adequate resident microglial population because diminished microglia are associated with decreased vascularity in models of ischemic retinopathy and retinal vascular development. In light of these findings, the traditional definition of microglia as merely immunocompetent cells should be reconsidered to encompass this new function related to blood vessel formation. (*Invest Ophthalmol Vis Sci.* 2006;47:3595–3602) DOI:10.1167/iovs.05-1522

Vascularization of the retina is a complex process involving astrocytes and endothelial cells (ECs) stimulated by the increasing oxygen (O_2) demands of differentiating neural cells.[1] Astrocytes enter the retina through the optic nerve[2] and distribute ahead of the growing vascular front,[3] forming a physical template on which ECs organize their vascular network.[4] Specialized ECs, termed tip cells, are located at the ends of the vascular sprouts and guide blood vessel growth through the tissue, whereas ECs of the blood vessel stalk proliferate to form patent capillaries.[5] In ischemic proliferative retinopathies (e.g., retinopathy of prematurity [ROP] and diabetic retinopathy), this organized retinal vessel formation is interrupted, and reductions in vascularity are followed by abnormal intravitreal neovascularization. However, the mechanisms necessary for proper intraretinal blood vessel formation and the factors in the ischemic retina that inhibit this process under pathologic conditions are not clearly understood.

Retinal microglia comprise a heterogeneous population of cells, believed to be of hemangioblastic mesodermal origin,[6] all which constitutively express CD45 (leukocyte common antigen) and major histocompatibility complex (MHC)-I and II, with a subpopulation also bearing macrophage-specific markers.[7,8] As such, they are considered specialized local immunocompetent cells. During human retinal development, microglia lacking macrophage markers are present by 10 weeks of gestation (WG), before astrocyte invasion and onset of vasculogenesis, whereas those positive for macrophage antigen (S22) appear at approximately 14 WG, the beginning of vascularization.[9] It has been suggested that this latter subpopulation represents vessel-associated perivascular microglia detected in the adult retina[8,10]; along these lines, microglia have been shown to extend their processes to orientate toward and contact blood vessels as they form.[11–13] However, the functional inference of these observations remains speculative.

Since the late 1970s, the macrophage has been recognized as a key angiogenic effector cell.[14–18] Macrophages induce angiogenesis by secreting factors that are pro-angiogenic, break down basement membrane, and stimulate other cells to produce greater amounts of pro-angiogenic substances.[14] In this context, microglia are also known to release a number of pro-angiogenic products, including growth factors (e.g., transforming growth factor-β,[19] basic fibroblast growth factor[20,21]), matrix metalloproteinases,[22,23] and cytokines (e.g., tumor necrosis factor [TNF]-α, interleukin-1[24,25]). Thus, although it is feasible that microglia, as resident central nervous system macrophages, may play roles similar to those of macrophages in blood vessel formation, little consideration has been given to such a possibility. We therefore hypothesized that microglia are involved in the formation of retinal blood vessels.

Herein we illustrate that microglial deficit leads to diminished blood vessel formation in the retina. The loss of microglia in two different models of ROP was shown to occur concomitantly with the phase characteristic of vascular reduction. In addition, in developmental retinal vascularization, pharmaco-

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logic depletion of resident microglia was also associated with decreased vascularity, an effect that intravitreal injection of microglia corrected. Together these observations highlight a novel function of microglia, broadening its designation as a specialized immunocompetent cell by disclosing its role in retinal blood vessel formation.

Materials and Methods

Human Samples

Fetal eyes were collected from terminated pregnancies after the receipt of informed, signed, parental consent according to guidelines approved by the Ethics Committee of Hôpital Ste. Justine. Subjects with ocular malformations or medical conditions related to vascular impairment were excluded.

Animals and Models of Retinopathy of Prematurity

The fractalkine (CX_3CL1) receptor CX_3CR1 is present on retinal microglia.[26] As such, eyes from postnatal day (P) 6 mice expressing green fluorescent protein (GFP) under the CX_3CR1 promoter ($CX_3CR1^{GFP/+}$) on a C57BL/6 background, as previously described,[27] were used to examine microglia with respect to developing retinal blood vessels.

A model of O_2-induced retinopathy[28] was used with slight modifications. C57BL/6 mice (Charles River, St. Constant, QC, Canada) were exposed to 75% O_2 (Oxycycler A820CV; BioSpherix Ltd., Redfield, NY) from P7 to P12, producing a loss of central retinal microvasculature.

Elevated arterial carbon dioxide (CO_2) has been identified as a risk factor for ROP.[29,30] A protocol of raised inspired CO_2 level, previously shown to retard normal retinal vascular development,[30,31] was followed with minor changes. Sprague-Dawley rats (Charles River) were exposed to 10% CO_2 within 24 hours of birth for 12 hours, a period sufficient to significantly reduce the vascularized area of the retina.[31]

Sprague-Dawley rats were used for all retinal explant cultures, clodronate liposome experiments, and cerebral microglial isolations, and Yorkshire piglets (Fermes Ménard, L'Ange-Gardien, QC, Canada) were used to derive retinal microvascular ECs.

All procedures were approved by the Animal Care Committee of Hôpital Ste. Justine and conformed with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal Wholemounts

Eyes were fixed in 4% paraformaldehyde. Retinas were dissected free, subjected to 100% methanol (−20°C) for 10 minutes, and incubated overnight (room temperature) in 1% Triton X-100/phosphate-buffered saline (PBS) with either the TRITC-conjugated lectin EC marker *Ulex europaeus* (1:100; Sigma-Aldrich, St. Louis, MO) and an antibody against the microglial marker CD18 (1:50; PharMingen, BD Biosciences, Mississauga, ON, Canada) (human samples) or the tetramethylrhodamine isothiocyanate (TRITC)–conjugated lectin EC/microglial marker *Griffonia simplicifolia*[11] (rodent samples) (1:100; Sigma-Aldrich). Double-labeled retinas were incubated with a monoclonal Alexa–conjugated secondary antibody (1:10 000; Molecular Probes, Burlington, ON, Canada) for 1 hour (room temperature) before all retinas were washed in PBS and mounted. Negative control experiments were performed in parallel by incubating retinas in 1% Triton X-100/PBS alone, followed by secondary antibody if one was used.

Retinal Explants

Retinal explant cultures were established as we and others have previously reported.[32–34] In short, P6 rats were euthanatized, eyes were enucleated, and retinas were immediately dissected free under aseptic conditions. Each retina was placed in a drop of 1% fetal bovine serum (FBS)/DMEM (Nuclepore Track-Etch Membrane; Whatman, Florham Park, NJ) resting on the surface of 6 mL of 1% FBS/DMEM in a six-well plate. The explants were cultured in a humidified 37°C atmosphere under standard (5% CO_2/21% O_2), high O_2 (5% CO_2/75% O_2), or high CO_2 (10% CO_2/21% O_2) culture conditions. The peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride (FeTPPS; Calbiochem, Mississauga, ON, Canada) and the superoxide dismutase (SOD) mimetic copper[II] [3,5-diisopropylsalicylate acid]$_2$ (CuDIPS; Calbiochem) were added to the media at experiment onset (final concentration, 10 μM). After 3 days, the explants (drug treated and controls) were fixed in 4% paraformaldehyde and prepared as described here with the general microglial marker OX-42 (1:50; Serotec, Raleigh, NC) followed by Alexa-conjugated monoclonal secondary antibody.

Clodronate Liposome Preparation

Clodronate liposomes elicit selective depletion of macrophages by apoptosis[35,36] and have been studied in systems demonstrating their insensitivity toward other cell types.[36–38] Thus, liposomes with entrapped clodronate were produced as detailed.[39] Briefly, 86 mg phosphatidylcholine (Sigma-Aldrich) and 8 mg cholesterol (Sigma-Aldrich) were dissolved in chloroform. After low vacuum rotary evaporation (37°C) of the chloroform phase, the lipid film was dispersed in 10 mL of a 0.6 M clodronic acid solution. After swelling, nonencapsulated clodronate was removed by centrifugation, and clodronate-containing liposomes were washed and resuspended in 4 mL sterile PBS.

Cell Isolation and Cell Death Assay

To ensure that clodronate liposomes do not affect EC viability, the effects of clodronate liposomes on EC survival were tested on cultured ECs. To obtain sufficient yields, primary ECs were derived from porcine retinal microvessels.[40] Microglia were isolated from early postnatal rat brain as previously described.[41] After 24-hour exposure to clodronate liposomes (5 μL), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich; 0.5 mg/mL in PBS) was incubated with cells for 2 hours (37°C). Media were then aspirated, the formazan product produced through the reduction of MTT by viable cells was solubilized with acidified isopropanol, and optical density was measured at 600 nm.

In Vivo Macrophage Depletion with Clodronate Liposomes and Intravitreal Injection of Microglia

Within 24 hours of birth (i.e., at the approximate onset of retinal vascularization), rats were injected intraperitoneally (60 μL) and intravitreally (4 μL; FemtoJet Microinjector; Eppendorf, Toronto, ON, Canada) with clodronate liposomes except where otherwise stated. Because liposomes cannot cross the blood/brain-retinal barrier,[42] intravitreal injection was necessary to deplete resident macrophages (i.e., retinal microglia). Administration of PBS alone, not liposomes containing PBS, is considered the proper control because comparisons should be made to animals with normal nonblocked, nonsuppressed, and nonactivated macrophages.[39] Rats were euthanatized and their eyes were enucleated 5, 6, and 8 days after clodronate liposome treatment. Eyes were processed for retinal wholemounting, as described here, or paraffin embedding and sectioning. The sections (7 μm) were cut sagittally, parallel to the optic nerve, and processed for periodic acid-Schiff and hematoxylin staining.

To add back microglia, on the day of the experiment microglial cells were trypsinized, counted, and resuspended in media to yield 10^5 cells/intravitreal injection (FemtoJet Microinjector; Eppendorf). Six days after clodronate liposome treatment (at P6), rats were injected intravitreally with media alone or microglia. At P10, all animals were euthanatized and retinal wholemounts were prepared, as described here.

Quantification Methods

Images were captured (Eclipse E800 microscope; Nikon, Tokyo, Japan); individual images were captured in the same plane. All measurements were made by two masked observers (Image-Pro Plus software, version 4.1; Media Cybernetic, Silver Spring, MD). Microglia of the

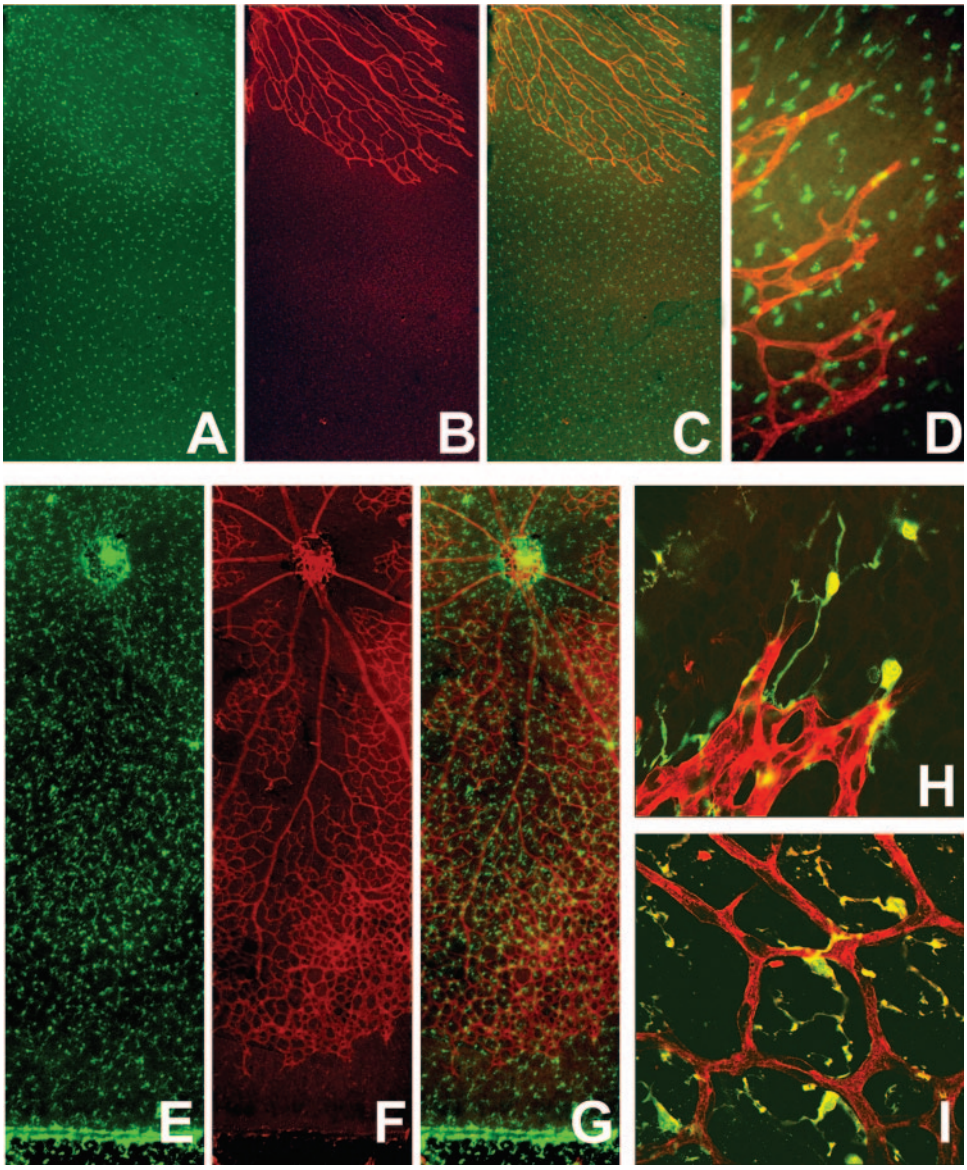

FIGURE 1. Retinal wholemount histochemistry illustrating the relationship between microglia and vasculature in the developing retina. (**A–D**) Human fetal retina (15 weeks of gestation) with lectin-stained vessels (*red*) and CD18-labeled microglia (*green*) ($n = 3$; representative images shown). Magnifications: (**A–C**) ×4; (**D**) ×20. (**E–I**) Six-day-old mice expressing green fluorescent protein driven by the fractalkine (CX_3CL1) receptor CX_3CR1 of retinal microglia ($n = 4$; representative images presented). Vessels were stained with lectin (*red*). Magnifications: (**E–G**) ×10; (**H–I**) ×40.

superficial layer were counted from three randomly selected 40× fields of view per retina. The surface area of the retina covered by vessels was measured and expressed as a percentage of the entire retinal area to obtain the vascularized area. Vessel density was determined by tracing all vessels present in three randomly selected vascularized areas and dividing the total length (in μm) by the total area (in μm^2). For uniformity, all vascularity measurements are presented as a percentage of the control.

Statistical Analysis

Data were analyzed, as required, by Student *t* test or one-way ANOVA with comparisons among the means performed by the appropriate post hoc test. Statistical significance was set at $P < 0.05$.

RESULTS

Microglia Ahead, and in Intimate Apposition, of Retinal Blood Vessels

In humans of 15 WG, retinal blood vessels are just beginning to emanate from the optic disc, whereas microglia already occupy the entire retinal surface (Figs. 1A–C). With the use of transgenic mice that expressed GFP specifically in microglial cells, we were able to visualize a similar topographic relationship because at P6 the microglial cells were present beyond the vascular front (Figs. 1E–G). Moreover, the GFP-positive microglia revealed, with unprecedented precision, the apposition of their processes with the endothelial tip cell filopodia and endothelial stalk cells (Fig. 1H). Throughout the developing retina, including the tip and stalk cells of the vascular front, all ECs appeared to be in intimate proximity of microglia (Figs. 1D, H and I).

Ischemic Retinopathy Models of Retinopathy of Prematurity Associated with Loss of Microglia

Models of ROP have been reproduced in animals exposed to hyperoxia and hypercapnia (augmented arterial CO_2)[28,30]; these factors elicit oxidative and nitrative stress, respectively, and contribute to ischemic proliferative retinopathies.[31,43,44] We determined the impact of such conditions on microglia in corresponding models of ROP. Retinal wholemounts from mice exposed to hyperoxia have a marked loss of microglia in the areas coinciding with central capillary dropout character-

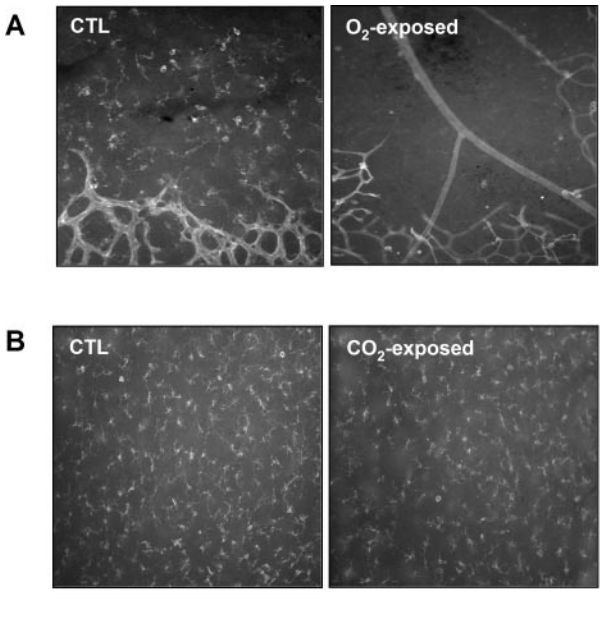

FIGURE 2. Microglial expression in models of retinopathy of prematurity. (**A**) C57BL/6 mice were exposed to hyperoxia (75% O_2) from P7 to P12. Representative retinal wholemounts (stained with lectin, which detects vessels and microglia[11]) before and after O_2 exposure are shown. Magnification: ×20. Histogram data represent means ± SEM of 20 O_2-exposed retinas and 10 control retinas. $^*P < 0.0001$ compared with control; Student *t* test. (**B**) Rats were exposed to hypercapnia (10% CO_2) from within 24 hours of birth for 12 hours. Representative retinal wholemounts (labeled with lectin) before and after CO_2 exposure are depicted. Magnification: ×20. Histogram data represent mean ± SEM of 6 to 9 retinas. $^*P = 0.0002$ compared with control; Student *t* test.

istic of this model (Fig. 2A); microglia were sparsely present in the remaining vascularized regions. Similarly, rats subjected to CO_2 exhibited significantly reduced microglial counts (Fig. 2B) when hypercapnia was shown to retard vessel growth.[31] It should be noted that because these rats were 36 hours old or younger, the extent of retinal vascularization was minimal (approximately 18% of surface); however attenuation of vascular development was clearly detected in these and in older animals.[30,31] Thus, an early or a late loss of microglia was associated with a coincidental decrease in retinal vascularity.

Free Radical Mediation of Microglial Loss

To corroborate the in vivo microglial loss and to elucidate a potential mechanism underlying this observation, ex vivo retinal explants were exposed to hyperoxic and hypercapnic conditions in the presence and absence of antioxidants (Fig. 3). The SOD mimetic CuDIPS was used to combat the reactive O_2 species superoxide in the O_2-exposed group, whereas the peroxynitrite decomposition catalyst FeTPPS was selected to minimize (protein) nitration observed in retinas exposed to high levels of CO_2.[31] A difference in microglia morphology was noted between hyperoxia- and hypercapnia-exposed tissues that might have been caused by cell activation or impending cell death (especially in the more severe hyperoxia-exposed tissues). Regardless, both antioxidants protected against microglial loss (Fig. 3), as reported for the vasculature.[31,44] Because of the close relationship between microglia and ECs, we could not establish the precise sequence of cell types affected in these retinopathy models; therefore, we proceeded to determine the

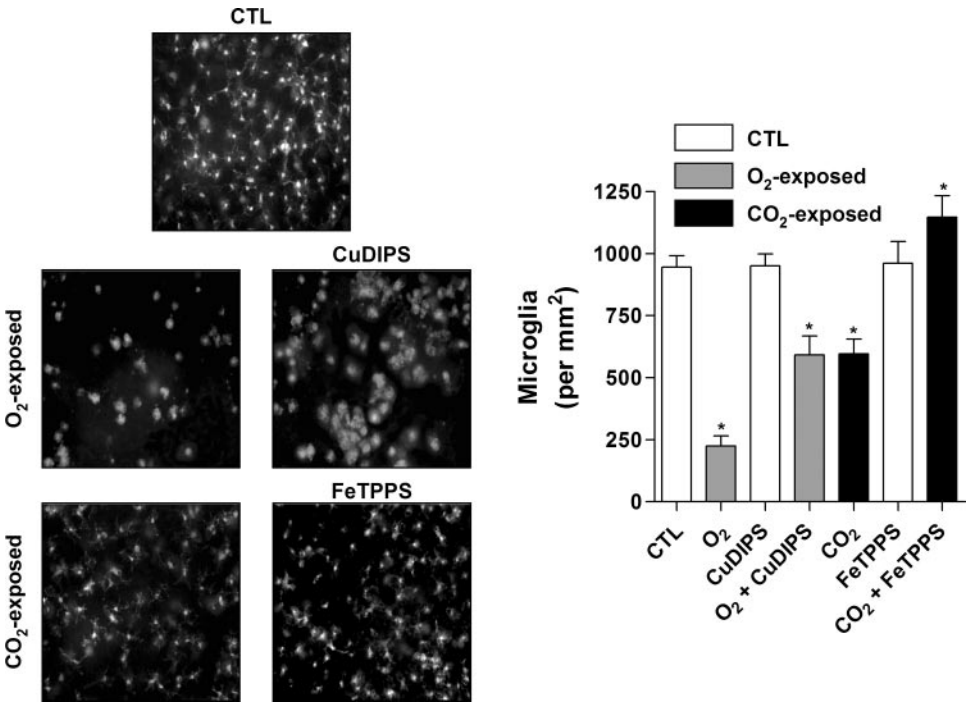

FIGURE 3. Microglial expression in ex vivo retinal explants. Retinas of P6 rats were isolated and cultured for 3 days under control, hyperoxic (75% O_2), or hypercapnic (10% CO_2) conditions, with or without the superoxide dismutase mimetic CuDIPS (10 μM) or the peroxynitrite decomposition catalyst FeTPPS (10 μM). After 3 days, retinas were stained with the general microglial marker OX-42, and positive cells were counted. Representative explants are presented. Magnification: ×40. Histogram data represent mean ± SEM of 9 to 15 retinas. $^*P < 0.001$ compared with appropriate control by one-way ANOVA followed by Bonferroni multiple comparison test.

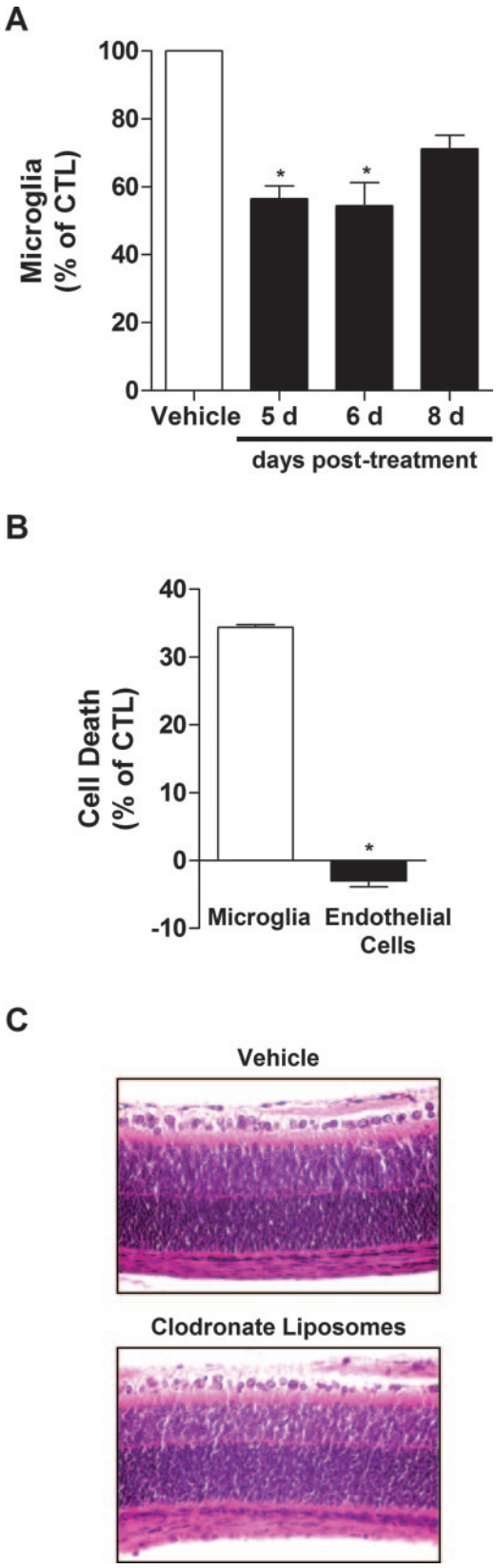

FIGURE 4. Clodronate liposome effectiveness and specificity in vivo and in vitro. (**A**, **C**) Within 24 hours of birth, rats were injected intravitreally and intraperitoneally with either vehicle or clodronate liposomes. (**A**) Histogram data of microglial counts from retinal whole-mounts labeled with lectin 5, 6, and 8 days after injection. Data are mean ± SEM of 12 retinas per group expressed as percentages relative to untreated controls. $^*P < 0.01$ compared with vehicle alone by one-way ANOVA followed by Tukey multiple comparison test. (**B**) Endothelial and microglial cells were exposed to clodronate liposomes (5 μL) for 24 hours before cell viability was assessed by MTT assay. Values represent mean ± SEM of three independent experiments expressed as percentages relative to controls. $^*P < 0.0000025$ compared with microglia group by Student *t* test. (**C**) Panels depict representative retinal sections 8 days after injection ($n = 3$). Magnification: ×40.

effect of microglial depletion on developmental retinal blood vessel formation.

Resident Macrophages Necessary for Normal Retinal Vascular Development

The impact of microglia on developmental retinal blood vessel formation was studied in neonatal rats undergoing retinal vascularization by depleting and subsequently intravitreally injecting retinas with microglia. Microglia were specifically diminished by clodronate liposomes (Figs. 4A, B), which had no negative effect on EC viability (in culture) (Fig. 4B). With the exception of the diminished presence of EC nuclei along the inner surface of the retina, the retinal structure was unaltered by the clodronate liposome treatment (Fig. 4C).

Five days after pharmacologic depletion of systemic macrophages and resident retinal microglia, pronounced decreases in retinal vascular area and density were observed (Figs. 5A, B). This effect persisted until at least 8 days after treatment, at which point there was a slight tendency toward recovery of vascular density (Figs. 5A, B). In addition, 8 days after treatment, the microglial count no longer significantly differed from that of the vehicle (Fig. 4A).

We distinguished the role of systemic macrophages from that of resident retinal microglia by administering clodronate liposomes either intraperitoneally or intravitreally. The retinal vascularity of rats receiving both injections was comparable to those receiving intravitreal injections alone (Fig. 6A). Similarly, retinal vascularity was indistinguishable between animals injected intraperitoneally with clodronate liposomes or PBS (Fig. 6A); intraperitoneal clodronate liposomes diminished systemic macrophages by approximately 70%,[45] indicating a prominent role for resident retinal microglia, as opposed to systemic macrophages, in normal retinal blood vessel formation. Moreover, intravitreal injection of microglia into microglia-depleted retinas rescued the diminished vascularity observed in clodronate-treated rats (Fig. 6B).

DISCUSSION

The contribution of macrophages to blood vessel formation is well recognized.[14,46,47] Although most studies focus on their angiogenic involvement in tumor development, wound healing, and inflammation, macrophages reportedly play a similar role in choroidal abnormalities[48,49] and in normal retinal development.[50] However, the participation of microglia in retinal blood vessel formation has received little attention to date. This study reveals that ischemic proliferative retinopathy is associated with a loss of resident microglia and that developmental retinal blood vessel growth and density are altered by specific pharmacologic microglial depletion (and subsequent intravitreal injection of microglia).

Microglia of the fetal and adult human retina are restricted to the inner vascularized regions (i.e., the nerve fiber/ganglion cell layer and the inner and outer plexiform layers),[8,51] appearing first before vascularization and then later along with the onset of vasculogenesis.[51] Yet the reason for their presence

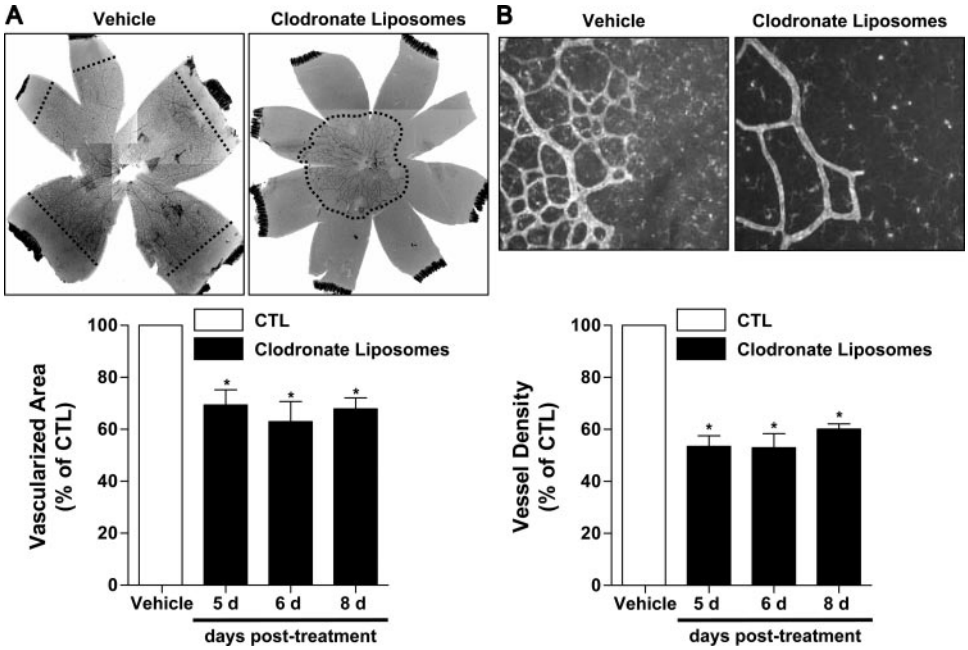

FIGURE 5. Effects of microglial and macrophage depletion on developmental retinal blood vessel formation. Intravitreal and intraperitoneal clodronate liposome injections were administered to rats within 24 hours of birth. Animals were euthanatized 5, 6, and 8 days after treatment, retinas were isolated and wholemounted to assess vascularity, and blood vessels were stained with lectin, which also detects microglia.[11] (**A**) Representative retinal wholemounts 6 days after treatment; retinal and ciliary microvessels are clearly detectable. *Dotted lines* delimit the retinal vascular front. Magnification: ×4. (**B**) Representative portions of retinal wholemounts depicting vessel density 6 days after treatment. Magnification: ×20. (**A**, **B**) Histogram data represent mean ± SEM of 8 to 17 retinas. $^*P < 0.001$ compared with control; one-way ANOVA followed by Bonferroni multiple comparison test.

remains speculative. It has been suggested that microglia are attracted to the retina to phagocytose the pyknotic debris that accompanies neural remodeling.[52] However, this process occurs through centrifugal orientation, whereas, on the contrary, microglia appear in greatest number at the periphery as the developing human retina becomes more abundant centrally throughout gestation.[51] Furthermore, because microglial invasion significantly precedes neuronal death,[11,51] microglial numbers increase beyond the peak of pyknosis,[51] and adjacent retinal cells—not macrophages—are largely responsible for engulfing most pyknotic cells,[53] it seems likely that microglia have other functions in retinal development.

In rat neural allografts, transplantation of neural cell suspensions into intact striatum reveals that microglia appear before blood vessels, after which they are in proximity with the vascular sprouts and become intimately associated with the ingrowing vasculature.[13] Our observations are consistent with these neural system findings: (1) The retina is populated with microglia before vascular development in rodents and humans (Figs. 1, 2). (2) Models of ischemic retinopathy induced by hyperoxia and hypercapnia reveal a loss of microglia that coincides with vasoattenuation (Figs. 2, 3). These retinal vasculopathy-inducing conditions[31,43,44] also evoke free radical–mediated damage against microglia (Fig. 3). (4) Depletion of microglia (using clodronate) during developmental retinal vascularization interferes with this process (Fig. 5). Endothelial cell demise is unlikely to affect microglia viability because clodronate does not elicit EC death (Fig. 4); this inference is consistent with other reports that, on the contrary, suggest a role for microglia in EC survival.[54,55] (5) Intravitreal injection of microglia into microglia-depleted retinas restores vascularity in the developing tissue (Fig. 6). Collectively, these observa-

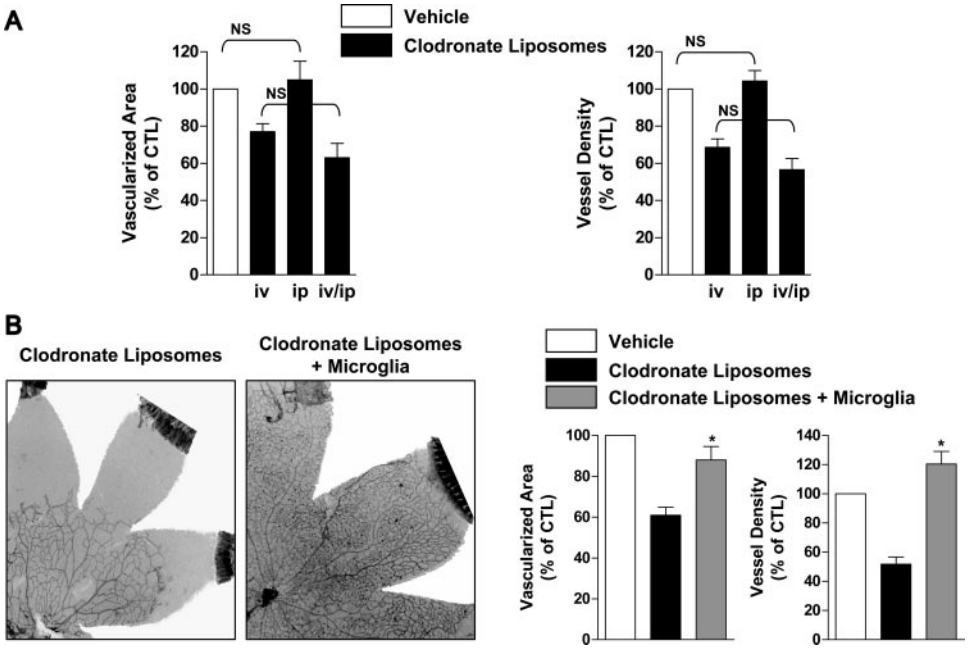

FIGURE 6. Effects of retinal microglial compared with systemic macrophage depletion on vascularity. Intravitreal (iv) and intraperitoneal (ip) clodronate liposomes were administered to rats within 24 hours of birth, and retinas were isolated, stained with lectin, and wholemounted to assess vascularity. (**A**) Histogram data represent mean ± SEM of 8 to 14 retinas 6 days after injection. $P < 0.01$ compared with all other groups by one-way ANOVA followed by Tukey multiple comparison test (with the exception of the nonsignificant [NS] comparisons noted). (**B**) Microglia was injected in rats 6 days after clodronate liposome treatment; microglia or vehicle was injected intravitreally. Animals were euthanatized at P10 (4 days after receiving microglia). Representative portions of retinal wholemounts are depicted. Magnification: ×10. Histogram data represent mean ± SEM of 8 to 18 retinas. $^*P < 0.0013$ compared with appropriate control by Student *t* test.

tions point to a significant involvement of microglia in retinal blood vessel formation.

Although the precise mediator(s) underlying the functional interaction between microglia and ECs in retinal blood vessel formation is unknown, one could surmise a possible role for a number of growth factors, matrix metalloproteinases, and cytokines produced by microglia. For instance, in O_2-induced retinopathy, hyperoxia has been shown to decrease proangiogenic TNF-α levels in the retina at P12,[56] which corresponds to the time of microglial and microvascular loss (Fig. 2A). Conversely, the ensuing hypoxia that follows the hyperoxic period in this model is associated with an elevation of microglial TNF-α and coincides with the intravitreal neovascular phase.[56,57] In addition, because microglia have been shown to migrate and proliferate in response to VEGF,[58] hyperoxia may prevent these effects by decreasing VEGF levels.

The effects of leukocytes on ocular blood vessel remodeling are complex. In contrast to findings presented herein, macrophages participate in the regression of transient vascular networks (such as the hyaloid), which are required only until permanent vessel beds are established.[59] Other leukocytes, specifically cytotoxic T lymphocytes, are also involved in retinal blood vessel remodeling and vaso-obliteration.[60] However, this does not imply that all leukocytes hinder all ocular vascular development; rather, divergent roles of macrophages in different settings have been proposed.[61,62] Hence, one can surmise that microglia are implicated in vascularization of the retina, whereas other macrophages and T lymphocytes act by pruning it.

In summary, our data suggest that local retinal microglia have an important role in organized blood vessel formation. Therefore, if microglia elicit angiogenesis when exposed to low O_2 tensions, as has been documented for macrophages,[18] it is tempting to propose their involvement in retinal blood vessel formation in response to local tissue hypoxia under physiologic[1] or pathologic[63] conditions.

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References

1. Chan-Ling T, Gock B, Stone J. The effect of oxygen on vasoformative cell division: evidence that 'physiological hypoxia' is the stimulus for normal retinal vasculogenesis. *Invest Ophthalmol Vis Sci*. 1995;36:1201–1214.
2. Watanabe T, Raff MC. Retinal astrocytes are immigrants from the optic nerve. *Nature*. 1988;332:834–837.
3. Ling TL, Mitrofanis J, Stone J. Origin of retinal astrocytes in the rat: evidence of migration from the optic nerve. *J Comp Neurol*. 1989;286:345–352.
4. Jiang B, Bezhadian MA, Caldwell RB. Astrocytes modulate retinal vasculogenesis: effects on endothelial cell differentiation. *Glia*. 1995;15:1–10.
5. Gerhardt H, Golding M, Fruttiger M, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol*. 2003; 161:1163–1177.
6. Streit WJ. Microglia and macrophages in the developing CNS. *Neurotoxicology*. 2001;22:619–624.
7. Penfold PL, Provis JM, Liew SC. Human retinal microglia express phenotypic characteristics in common with dendritic antigen-presenting cells. *J Neuroimmunol*. 1993;45:183–191.
8. Provis JM, Penfold PL, Edwards AJ, van Driel D. Human retinal microglia: expression of immune markers and relationship to the glia limitans. *Glia*. 1995;14:243–256.
9. Diaz-Araya CM, Provis JM, Penfold PL. Ontogeny and cellular expression of MHC and leucocyte antigens in human retina. *Glia*. 1995;15:458–470.
10. Provis JM, Diaz CM, Penfold PL. Microglia in human retina: a heterogeneous population with distinct ontogenies. *Perspect Dev Neurobiol*. 1996;3:213–222.
11. Ashwell KW, Hollander H, Streit W, Stone J. The appearance and distribution of microglia in the developing retina of the rat. *Vis Neurosci*. 1989;2:437–448.
12. Ling EA. A light microscopic demonstration of amoeboid microglia and microglial cells in the retina of rats of various ages. *Arch Histol Jpn*. 1982;45:37–44.
13. Pennell NA, Streit WJ. Colonization of neural allografts by host microglial cells: relationship to graft neovascularization. *Cell Transplant*. 1997;6:221–230.
14. Lingen MW. Role of leukocytes and endothelial cells in the development of angiogenesis in inflammation and wound healing. *Arch Pathol Lab Med*. 2001;125:67–71.
15. Polverini PJ, Cotran PS, Gimbrone MA Jr, Unanue ER. Activated macrophages induce vascular proliferation. *Nature*. 1977;269: 804–806.
16. Sunderkotter C, Goebeler M, Schulze-Osthoff K, Bhardwaj R, Sorg C. Macrophage-derived angiogenesis factors. *Pharmacol Ther*. 1991;51:195–216.
17. Crowther M, Brown NJ, Bishop ET, Lewis CE. Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *J Leukoc Biol*. 2001;70:478–490.
18. Knighton DR, Hunt TK, Scheuenstuhl H, et al. Oxygen tension regulates the expression of angiogenesis factor by macrophages. *Science*. 1983;221:1283–1285.
19. Lu YZ, Lin CH, Cheng FC, Hsueh CM. Molecular mechanisms responsible for microglia-derived protection of Sprague-Dawley rat brain cells during in vitro ischemia. *Neurosci Lett*. 2005;373:159–164.
20. Shimojo M, Nakajima K, Takei N, Hamanoue M, Kohsaka S. Production of basic fibroblast growth factor in cultured rat brain microglia. *Neurosci Lett*. 1991;123:229–231.
21. Liu X, Mashour GA, Webster HF, Kurtz A. Basic FGF and FGF receptor 1 are expressed in microglia during experimental autoimmune encephalomyelitis: temporally distinct expression of midkine and pleiotrophin. *Glia*. 1998;24:390–397.
22. Cross AK, Woodroofe MN. Chemokine modulation of matrix metalloproteinase and TIMP production in adult rat brain microglia and a human microglial cell line in vitro. *Glia*. 1999;28:183–189.
23. Gottschall PE, Deb S. Regulation of matrix metalloproteinase expressions in astrocytes, microglia and neurons. *Neuroimmunomodulation*. 1996;3:69–75.
24. Nagai A, Nakagawa E, Hatori K, et al. Generation and characterization of immortalized human microglial cell lines: expression of cytokines and chemokines. *Neurobiol Dis*. 2001;8:1057–1068.
25. Chao CC, Hu S, Close K, et al. Cytokine release from microglia: differential inhibition by pentoxifylline and dexamethasone. *J Infect Dis*. 1992;166:847–853.
26. Carter DA, Dick AD. CD200 maintains microglial potential to migrate in adult human retinal explant model. *Curr Eye Res*. 2004;28:427–436.
27. Jung S, Aliberti J, Graemmel P, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol*. 2000;20:4106–4114.
28. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci*. 1994;35:101–111.
29. Shohat M, Reisner SH, Krikler R, et al. Retinopathy of prematurity: incidence and risk factors. *Pediatrics*. 1983;72:159–163.
30. Holmes JM, Leske DA, Zhang S. The effect of raised inspired carbon dioxide on normal retinal vascular development in the neonatal rat. *Curr Eye Res*. 1997;16:78–81.
31. Checchin D, Sennlaub F, Sirinyan M, et al. Hypercapnia prevents neovascularization via nitrative stress. *Free Radic Biol Med*. 2006; 40:543–553.
32. Altshuler D, Lo Turco JJ, Rush J, Cepko C. Taurine promotes the differentiation of a vertebrate retinal cell type in vitro. *Development*. 1993;119:1317–1328.
33. Hatakeyama J, Kageyama R. Retrovirus-mediated gene transfer to retinal explants. *Methods*. 2002;28:387–395.

34. Kermorvant-Duchemin E, Sennlaub F, Sirinyan M, et al. Transarachidonic acids generated during nitrative stress induce a thrombospondin-1-dependent microvascular degeneration. *Nat Med*. 2005;11:1339–1345.
35. Naito M, Nagai H, Kawano S, et al. Liposome-encapsulated dichloromethylene diphosphonate induces macrophage apoptosis in vivo and in vitro. *J Leukoc Biol*. 1996;60:337–344.
36. Schmidt-Weber CB, Rittig M, Buchner E, et al. Apoptotic cell death in activated monocytes following incorporation of clodronate-liposomes. *J Leukoc Biol*. 1996;60:230–244.
37. Bogers WM, Stad RK, Janssen DJ, et al. Kupffer cell depletion in vivo results in clearance of large-sized IgA aggregates in rats by liver endothelial cells. *Clin Exp Immunol*. 1991;85:128–136.
38. Diez-Roux G, Lang RA. Macrophages induce apoptosis in normal cells in vivo. *Development*. 1997;124:3633–3638.
39. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods*. 1994;174:83–93.
40. Lahaie I, Hardy P, Hou X, et al. A novel mechanism for vasoconstrictor action of 8-isoprostaglandin F2 alpha on retinal vessels. *Am J Physiol*. 1998;274:R1406–R1416.
41. Armstrong RC. Isolation and characterization of immature oligodendrocyte lineage cells. *Methods*. 1998;16:282–292.
42. Huitinga I, Damoiseaux JG, van Rooijen N, Dopp EA, Dijkstra CD. Liposome mediated affection of monocytes. *Immunobiology*. 1992;185:11–19.
43. Hardy P, Dumont I, Bhattacharya M, et al. Oxidants, nitric oxide and prostanoids in the developing ocular vasculature: a basis for ischemic retinopathy. *Cardiovasc Res*. 2000;47:489–509.
44. Beauchamp MH, Sennlaub F, Speranza G, et al. Redox-dependent effects of nitric oxide on microvascular integrity in oxygen-induced retinopathy. *Free Radic Biol Med*. 2004;37:1885–1894.
45. Masson MJ, Uetrecht JP. Tolerance induced by low dose D-penicillamine in the brown Norway rat model of drug-induced autoimmunity is immune-mediated. *Chem Res Toxicol*. 2004;17:82–94.
46. Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C. Macrophages and angiogenesis. *J Leukoc Biol*. 1994;55:410–422.
47. Polverini PJ. How the extracellular matrix and macrophages contribute to angiogenesis-dependent diseases. *Eur J Cancer*. 1996; 32A:2430–2437.
48. Sakurai E, Anand A, Ambati BK, van Rooijen N, Ambati J. Macrophage depletion inhibits experimental choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2003;44:3578–3585.
49. Pollack A, Korte GE, Heriot WJ, Henkind P. Ultrastructure of Bruch's membrane after krypton laser photocoagulation, II: repair of Bruch's membrane and the role of macrophages. *Arch Ophthalmol*. 1986;104:1377–1382.
50. Penfold PL, Provis JM, Madigan MC, van Driel D, Billson FA. Angiogenesis in normal human retinal development: the involvement of astrocytes and macrophages. *Graefes Arch Clin Exp Ophthalmol*. 1990;228:255–263.
51. Diaz-Araya CM, Provis JM, Penfold PL, Billson FA. Development of microglial topography in human retina. *J Comp Neurol*. 1995;363: 53–68.
52. Hume DA, Perry VH, Gordon S. Immunohistochemical localization of a macrophage-specific antigen in developing mouse retina: phagocytosis of dying neurons and differentiation of microglial cells to form a regular array in the plexiform layers. *J Cell Biol*. 1983;97:253–257.
53. Penfold PL, Provis JM. Cell death in the development of the human retina: phagocytosis of pyknotic and apoptotic bodies by retinal cells. *Graefes Arch Clin Exp Ophthalmol*. 1986;224:549–553.
54. Lin B, Ginsberg MD, Busto R, Dietrich WD. Sequential analysis of subacute and chronic neuronal, astrocytic and microglial alterations after transient global ischemia in rats. *Acta Neuropathol (Berl)*. 1998;95:511–523.
55. Siren AL, McCarron R, Wang L, et al. Proinflammatory cytokine expression contributes to brain injury provoked by chronic monocyte activation. *Mol Med*. 2001;7:219–229.
56. Yossuck P, Yan Y, Tadesse M, Higgins RD. Dexamethasone alters TNF-alpha expression in retinopathy. *Mol Genet Metab*. 2001;72: 164–167.
57. Yoshida S, Yoshida A, Ishibashi T. Induction of IL-8, MCP-1, and bFGF by TNF-alpha in retinal glial cells: implications for retinal neovascularization during post-ischemic inflammation. *Graefes Arch Clin Exp Ophthalmol*. 2004;242:409–413.
58. Forstreuter F, Lucius R, Mentlein R. Vascular endothelial growth factor induces chemotaxis and proliferation of microglial cells. *J Neuroimmunol*. 2002;132:93–98.
59. Lang RA, Bishop JM. Macrophages are required for cell death and tissue remodeling in the developing mouse eye. *Cell*. 1993;74:453–462.
60. Ishida S, Yamashiro K, Usui T, et al. Leukocytes mediate retinal vascular remodeling during development and vaso-obliteration in disease. *Nat Med*. 2003;9:781–788.
61. Duffield JS, Forbes SJ, Constandinou CM, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest*. 2005;115:56–65.
62. Lobov IB, Rao S, Carroll TJ, et al. WNT7b mediates macrophage-induced programmed cell death in patterning of the vasculature. *Nature*. 2005;437:417–421.
63. Pe'er J, Shweiki D, Itin A, et al. Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularizing ocular diseases. *Lab Invest*. 1995;72: 638–645.