Myeloid-Derived Vascular Endothelial Growth Factor and Hypoxia-Inducible Factor Are Dispensable for Ocular Neovascularization—Brief Report


Objective—Ocular neovascularization (ONV) is a pathological feature of sight-threatening human diseases, such as diabetic retinopathy and age-related macular degeneration. Macrophage depletion in mouse models of ONV reduces the formation of pathological blood vessels, and myeloid cells are widely considered an important source of the vascular endothelial growth factor A (VEGF). However, the importance of VEGF or its upstream regulators hypoxia-inducible factor-1α (HIF1α) and hypoxia-inducible factor-2α (HIF2α) as myeloid-derived regulators of ONV remains to be determined.

Approach and Results—We used 2 mouse models of ONV, choroidal neovascularization and oxygen-induced retinopathy, to show that Vegfa is highly expressed by several cell types, but not myeloid cells during ONV. Moreover, myeloid-specific VEGF ablation did not reduce total ocular VEGF during choroidal neovascularization or oxygen-induced retinopathy. In agreement, the conditional inactivation of Vegfa, Hif1α, or Epas1 in recruited and resident myeloid cells that accumulated at sites of neovascularization did not significantly reduce choroidal neovascularization or oxygen-induced retinopathy.

Conclusions—The finding that myeloid cells are not a significant local source of VEGF in these rodent models of ONV suggests that myeloid function in neovascular eye disease differs from skin wound healing and other neovascular pathologies. (Arterioscler Thromb Vasc Biol. 2016;36:19-24. DOI: 10.1161/ATVBAHA.115.306681.)

Key Words: choroidal neovascularization □ diabetic retinopathy □ hypoxia inducible factor □ macular degeneration □ myeloid cells □ retinal neovascularization □ vascular endothelial growth factor A

Myeloid-derived vascular endothelial growth factor (VEGF) has been proposed to drive ocular neovascularization (ONV),1,4 a pathological feature common to leading causes of blindness, including retinopathy of prematurity in infants, proliferative diabetic retinopathy in the working population, and age-related macular degeneration in the elderly.5 In mice with oxygen-induced retinopathy (OIR), a model of retinopathy of prematurity, VEGF-expressing macrophages are recruited to sites of retinal neovascularization (RNV), and clodronate-induced or genetic macrophage depletion reduces RNV, raising the possibility that myeloid-derived VEGF promotes RNV.5,6 In laser-induced choroidal neovascularization (CNV), a mouse model of age-related macular degeneration—associated neovascularization, peak VEGF expression correlates with maximal myeloid infiltration, and clodronate-induced macrophage depletion reduces both VEGF levels and CNV area.1 The absence of VEGF-producing CCR2+ macrophages also reduces CNV area.2 Human CNV lesions have also been reported to contain VEGF-expressing macrophages, which were suggested to cooperate with VEGF-expressing retinal pigment epithelium (RPE) to drive angiogenesis.3 These findings raised the possibility that myeloid-derived VEGF also promotes CNV. However, others contested that myeloid-derived VEGF enhances CNV.9 The significance of myeloid-derived VEGF in ONV, therefore, remains controversial. Moreover, the importance of myeloid-derived hypoxia-inducible factors, HIF1α and HIF2α, has not yet been defined for ONV, even though they regulate VEGF expression,10 have been implicated in myeloid-mediated angiogenesis in various tissues11 and are expressed in OIR.

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VEGF levels and CNV area.1 The absence of VEGF-producing CCR2+ macrophages also reduces CNV area.2 Human CNV lesions have also been reported to contain VEGF-expressing macrophages, which were suggested to cooperate with VEGF-expressing retinal pigment epithelium (RPE) to drive angiogenesis.3 These findings raised the possibility that myeloid-derived VEGF also promotes CNV. However, others contested that myeloid-derived VEGF enhances CNV.9 The significance of myeloid-derived VEGF in ONV, therefore, remains controversial. Moreover, the importance of myeloid-derived hypoxia-inducible factors, HIF1α and HIF2α, has not yet been defined for ONV, even though they regulate VEGF expression,10 have been implicated in myeloid-mediated angiogenesis in various tissues11 and are expressed in OIR.
and CNV models.\textsuperscript{12,13} To test the prevailing idea in the current literature that myeloid VEGF is nonredundant with other VEGF sources in ONV, we used conditional mouse knockout models to target Vegfa and its upstream regulators, Hif1α and Epas1 (Hif2α), in myeloid cells, and analyzed the effects of their deletion on RNV and CNV. Unexpectedly, we found that myeloid-derived HIFs and VEGF are dispensable ONV, suggesting that they do not present useful targets for therapy of ocular disease.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

Briefly, animal procedures were conducted with ethical approval under institutional and UK Home Office guidelines using Lysm\textsuperscript{+/Cre};Rosa26\textsuperscript{mT/mG} and Lysm\textsuperscript{+/Cre};Vegfa\textsuperscript{fl/fl} mice, previously shown to faithfully report Vegfa expression in macrophages and other cell types.\textsuperscript{11,14,15} X-gal staining of eye sections indicates prominent Vegfa expression in the RPE, inner nuclear layer, and retinal ganglion cell layer on postnatal day (P) 17 in the OIR and on day (D) 3 postlasering in the CNV model, when VEGF levels and myeloid infiltration peak,\textsuperscript{1,2,3} but Vegfa expression was below the detection limit in IBA1\textsuperscript{+} F4/80\textsuperscript{+} microglia/macrophages (Figure 1A and 1B). Vegfa expression was also undetectable in YFP\textsuperscript{+} IB4\textsuperscript{+} myeloid cells by in situ hybridization on D3 after laser in Lysm\textsuperscript{+/Cre} eyes carrying the Rosa26\textsuperscript{ERT2} reporter to identify myeloid cells, even though other cell types strongly expressed Vegfa (Figure 1C). These findings suggest that, compared with other ocular cell types, myeloid cells are unlikely a significant local source of Vegfa for ONV.

OIR retinas from Lysm\textsuperscript{+/Cre};Rosa26\textsuperscript{ERT2} myeloid reporter mice accumulated YFP\textsuperscript{+} myeloid cells in both avascular and vascularized areas at P14, before the onset of RNV (Figure 1D–1F). By P17, YFP\textsuperscript{+} myeloid cells had accumulated near neovascular tufts (Figure 1D, 1E, and 1G). We also observed YFP-expressing myeloid cells at sites of laser injury in Lysm\textsuperscript{+/Cre};Rosa26\textsuperscript{ERT2} eyes on D3 postlasering, the onset of CNV (Figure 1H). Flow cytometry analysis of D3 Lysm\textsuperscript{+/Cre};Rosa26\textsuperscript{ERT2} choroid/RPE complex showed efficient recombination in infiltrating CD11b\textsuperscript{+} myeloid cells, particularly neutrophils and inflammatory monocytes/macrophages (Figure 1I). Importantly, the Lysm\textsuperscript{+/Cre} allele did not affect the size of avascular or neovascular areas on P17 (Lysm\textsuperscript{+/Cre}; 6.9±1.9%; Lysm\textsuperscript{+/i}; 7.6±2.8%; mean±SD, n=15, P>0.05) or CNV lesions on D7 or D14 postlasering (Figure 1J). Lysm\textsuperscript{+/Cre} is therefore a suitable tool to target genes in myeloid cells recruited to sites of ONV.

Next, we examined Lysm\textsuperscript{+/Cre};Vegfa\textsuperscript{fl/fl} mice, which are deficient in myeloid cell–derived Vegfa and were previously shown to have reduced pathological angiogenesis in wound healing and cancer models.\textsuperscript{2,24} Lysm\textsuperscript{+/Cre};Vegfa\textsuperscript{fl/fl} mice appeared healthy as previously reported and had normal retinal angiogenesis (Figure IA in the online-only Data Supplement). YFP-expressing splenic myeloid cells showed efficient Vegfa gene targeting and, accordingly, Vegfa mRNA was reduced in mutant compared with control YFP\textsuperscript{+} splenic myeloid cells (Figure 2A and 2B). Nevertheless, myeloid Vegfa deletion did not alter overall VEGF protein or mRNA levels in the P17 OIR retina or D3 postlasering RPE/choroid (Figure 2C and 2D). In agreement, the size of the central avascular and neovascular areas in P17 OIR retina and D7 and D14 CNV lesions was similar in Lysm\textsuperscript{+/Cre};Vegfa\textsuperscript{fl/fl} mice and controls (Figure 2E–2F). Moreover, myeloid Vegfa depletion did not affect CD11b\textsuperscript{+} cell recruitment to the RPE/choroid on D3 postlasering (Figure 2G).

We also examined Tie2-Cre;Vegfa\textsuperscript{fl/fl} mice because Tie2-Cre targets yolk sac–derived tissue-resident macrophages more efficiently than Lysm\textsuperscript{+/Cre}, including microglia in the brain\textsuperscript{25,26} and retina (Figure 2H and 2I). Tie2-Cre;Vegfa\textsuperscript{fl/fl} mutant mice are healthy, and despite targeting of Vegfa in hematopoietic and endothelial cells, have no obvious vascular defects and only develop vascular dysfunction in old age.\textsuperscript{16,27} In agreement, angiogenesis and the density of resident myeloid cells were similar in mutant and control postnatal retinas (Figure IB and IC in the online-only Data Supplement). Moreover, the size of the central avascular and neovascular areas in P17 OIR retina and CNV lesions was not significantly different between mutants and controls (Figure 2J–2K). These data suggest that VEGF expression by resident microglia/macrophages does not explain the lack of angiogenesis defects in mice with Lysm\textsuperscript{+/Cre}-mediated targeting of Vegfa in myeloid cells. Myeloid cell–derived Vegfa is therefore dispensable for retinal angiogenesis and pathological ONV.

Because HIFs promote the expression of Vegfa and other hypoxia-induced proangiogenic molecules,\textsuperscript{10} we also targeted the genes encoding HIF1α and HIF2α in myeloid cells with Lysm\textsuperscript{+/Cre}. Targeting of Hif1α, Epas1, or both did not affect retinal vascular development, despite efficient Lysm\textsuperscript{+/Cre}-mediated Hif1α or Epas1 deletion in myeloid cells (Figure IIA and IIB in the online-only Data Supplement). Moreover, the size of the central avascular and neovascular areas on P17 after OIR (Figure IIIA and IIIB in the online-only Data Supplement) and D7 and D14 CNV lesions (Figure IIIC and IID in the online-only Data Supplement) were similar in controls and mutants for Hif1α, Epas1, or both. The recruitment of myeloid cells, including individual subpopulations, to ONV sites was also not impaired after Lysm\textsuperscript{+/Cre}-mediated targeting of Hif1α, Epas1, or both (Figure IIIE and IIIF in the online-only Data Supplement).

**Discussion**

Nonmyeloid VEGF is thought to promote RNV because retinal ganglion cells\textsuperscript{28,29} and Mueller cells\textsuperscript{30–32} are abundant VEGF...
sources in the OIR model. Moreover, it was shown that the deletion of Mueller cell–derived VEGF in a mouse model of diabetes mellitus reduces RNV.33 Furthermore, RPE-derived VEGF has been implicated in CNV in both mice34–36 and patients,8 and HIF1α depletion in RPE cells impairs VEGF expression and reduces CNV in mice.36,37 VEGF expression and myeloid cell depletion studies have been interpreted as evidence that myeloid-derived VEGF provides an additional, nonredundant source of VEGF for both RNV and CNV.1–4,8 However, our studies show that myeloid expression of VEGF or its upstream regulators, HIF1α and HIF2α, is not necessary for ONV in rodent models of OIR and CNV. Previous studies deducing a role for myeloid-derived VEGF in ONV by correlating the phenotype caused by myeloid cell depletion with changes in VEGF levels1–3 may, therefore, have only identified an indirect association of both pathological parameters in eye disease. For example, myeloid cells may influence ONV indirectly by stimulating VEGF production by other cell types, such as the...
Figure 2. Myeloid-derived Vegfa does not significantly contribute to the total vascular endothelial growth factor (VEGF) pool or ocular neovascularization. (A and B) Polymerase chain reaction detection of Vegfa gene (A) and mRNA recombination (B) in YFP+ splenocytes in Vegfa\textsuperscript{fl/fl};Lysm\textsuperscript{+/Cre};Rosa26\textsuperscript{Yfp} mutants (A and B) and Vegfa\textsuperscript{+/+}Lysm\textsuperscript{+/Cre};Rosa26\textsuperscript{Yfp} controls (B); n≥3 mice each, P<0.05, t test. (C and D) VEGF protein levels (C) in the P17 oxygen-induced retinopathy (OIR) retina (left) and the retinal pigment epithelium (RPE)/choroid on D3 after laser injury (right) and Vegfa mRNA (mean fold change relative to Actb, (D) in the RPE/choroid on D3 after laser injury in Lysm\textsuperscript{Cre};Vegfa\textsuperscript{fl/fl} mice and control littermates; mean±SD, n≥3 each; P>0.05, t test. (E–E”) IB4 staining (E) of P17 OIR Lysm\textsuperscript{Cre};Vegfa\textsuperscript{fl/fl} and control retina. E’, Total retina, avascular (AV) and neovascular (NV) areas are rendered gray, yellow, and red, respectively. E”. Proportion of central AV and NV areas; mean±SD, n≥6 each; P>0.05, t test. F–G, D14 angiograms (F) and choroidal neovascularization (CNV) lesion area on D7 and D14 (F’) and percentage of CD11b\textsuperscript{+} cells in choroid/RPE on D3 after laser injury (G) of Lysm\textsuperscript{Cre};Vegfa\textsuperscript{fl/fl} and control mice; mean±SD, n≥4 each; P>0.05, t test. H and I, Wholemount retina staining for IB4, F4/80, and YFP shows recombination in microglia in Lysm\textsuperscript{Cre};Rosa26\textsuperscript{Yfp} mice (H) and in most microglia and endothelium in Tie2-Cre;Rosa26\textsuperscript{Yfp} mice (I). J–J”, IB4 staining (J) of P17 OIR Tie2-Cre;Vegfa\textsuperscript{fl/fl} and control retina. J’. Total retina, AV, and NV areas are rendered gray, yellow, and red, respectively. J”. Proportion of central AV and NV areas in Tie2-Cre;Vegfa\textsuperscript{fl/fl} and control P17 OIR retina stained with IB4; mean±SD, n≥5 mice each; P>0.05, t test. K and K’, D14 angiograms (K) and quantification of CNV lesion area on D7 and D14 after laser injury (K) in Tie2-Cre;Vegfa\textsuperscript{fl/fl} mice and littermate controls; n≥5 mice each, P>0.05, t test. Scale bars, 1 mm (E, F, J, and K), 200 μm (H and I).
neural or glial sources previously implicated in ONV. Myeloid cells have also been found to influence angiogenesis by VEGF-independent mechanisms, for example, by acting as cellular chaperones to promote endothelial tip cell fusion during vascular development or by producing proangiogenic factors different from VEGF during tumor vascularization. The molecular mechanisms of inflammatory cell modulation of neovascular eye disease, therefore, differs significantly from nonocular disease models, in which myeloid-derived VEGF is nonredundant with other VEGF sources to promote pathological angiogenesis, even when nonmyeloid VEGF is abundant, for example, during tumor vascularization or in skin wound healing. 

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Disclosures

U.F. Luhmann is an employee of F. Hoffmann-La Roche Ltd. The other authors report no conflicts.

References


Previous work inferred from correlative studies that myeloid-derived vascular endothelial growth factor drives ocular neovascularization via induction of angiogenesis. Unexpectedly, we find that *Vegfa* is not expressed at significant levels by myeloid cells in the eye, and, accordingly, myeloid-derived vascular endothelial growth factor and its upstream regulator hypoxia-inducible factors are not required for ocular neovascularization. Our work implies organ-specific mechanisms by which myeloid cells regulate angiogenesis because myeloid cells do provide a significant and nonredundant source of vascular endothelial growth factor to promote pathological angiogenesis in other settings, such as skin wound healing and cancer. Moreover, our work suggests that understanding the role of myeloid cells in ocular angiogenesis requires focus on pathways unrelated to vascular endothelial growth factor or hypoxia-inducible factors.
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Supplemental Figure I. Myeloid-derived VEGF is not essential for developmental angiogenesis.

(A) Wholemount P7 Lysm^{Cre};Vegfa^{fl/fl} mutant and littermate control retinas, stained for alpha smooth muscle actin (SMA) together with IB4; the black and white panel show higher magnification of the areas indicated with white squares.

(B,C) Wholemount P7 retinas (B) from Tie2-Cre;Vegfa^{fl/fl} mutants and littermate controls stained for IBA1 together with IB4 show a similar density of IBA1^{+} microglia and similar vascular development; the black and white panel show higher magnification of the areas indicated with white squares. There is no significant difference in vascular extension across the retina between both genotypes (C); n ≥ 5 mice each, P>0.05, t-test.

Scale bars: 1 mm (A,B).
Supplemental Figure II. Myeloid-derived HIF is not essential for developmental angiogenesis.

(A) IB4 staining of wholemount P11 retinas shows similar vascular development in myeloid Hif1a-, Epas1- and double Hif1a- Epas1-deficient mice compared to littermate controls. 

(B) qPCR quantification of Hif1a and Epas1 genomic DNA levels in Hif1a-, Epas1-, double Hif1a-Epas1-deficient and control peritoneal neutrophils revealed efficient excision of Hif1a and Epas1 in Cre-expressing cells; mean±SD, n ≥ 3 mice; **P<0.01, ***P<0.001, 1-way ANOVA. 
Scale bar: 200 μm (A).
Supplemental Figure III. *Hif1a* and *Epas1* expression by myeloid cells is not necessary for ONV.

(A,B) Representative images of retinal flatmounts (A) from mice with *Lysm<sup>+/Cre</sup>*-mediated myeloid targeting of *Hif1a* and/or *Epas1* and controls, stained with IB4 on P17 in the OIR model; in the bottom panels, total retina, avascular (AV) and neovascular (NV) areas are colour-rendered grey, yellow and red, respectively. (B) Quantification of AV and NV areas on P17 in the OIR model for mice with *Lysm<sup>+/Cre</sup>*-mediated myeloid targeting of *Hif1a* and/or *Epas1* and controls; mean±SD, n ≥ 10 mice each; P>0.05, 1-way ANOVA.

(C,D) Fundus fluorescein angiogram on D14 (C) and quantification of average lesion area on D7 and D14 (D) after laser injury for mice with *Lysm<sup>+/Cre</sup>*-mediated myeloid targeting of *Hif1a* and/or *Epas1* and controls; mean±SD, n ≥ 10 mice each; P>0.05, 1-way ANOVA.

(E) Quantification of green fluorescent myeloid cells recruited to NV areas of *Lysm<sup>+/Cre</sup>;Rosa26<sup>mT/mG</sup>* retinas on P17 in the OIR model; n ≥ 4 mice each, P>0.05 1-way ANOVA.

(E') Flow cytometric analysis of the choroid/RPE complex on D3 after laser injury of mice with *Lysm<sup>+/Cre</sup>*-mediated myeloid targeting of *Hif1a* and/or *Epas1* and controls carrying the *Rosa26<sup>mT/mG</sup>* recombination reporter allele, showing the total number of CD11b<sup>+</sup> myeloid cells and myeloid cell subpopulations, in addition to CD11b<sup>+</sup> NK cells (left), and the percentage of each myeloid subset expressing GFP (right); mean±SD, n ≥ 5 mice each; P>0.05, 1-way ANOVA.

Scale bars: 1 mm (A,C).
Materials and Methods

Animals
Animal procedures were conducted with ethical approval under institutional and UK Home Office guidelines. Lysm\(^{+/\text{Cre}}\) mice were crossed to Hif1a\(^{\text{fl/fl}}\), Hif2a\(^{\text{fl/fl}}\) or Vegfa\(^{\text{fl/fl}}\) mice to generate Lysm\(^{+/\text{Cre}},\text{Hif1a}^{\text{fl/fl}},\) Lysm\(^{+/\text{Cre}},\text{Hif2a}^{\text{fl/fl}}\) and Lysm\(^{+/\text{Cre}},\text{Vegfa}^{\text{fl/fl}}\) mutants. In some experiments, we used Tie2-Cre;Vegfa\(^{\text{fl/fl}}\) mice. Vegfa\(^{\text{fl/+}}\)/LacZ or floxed Rosa26\(^{\text{Yfp}}\) and Rosa26\(^{\text{mT/mG}}\) were used as reporters of gene expression and Cre recombination, respectively. OIR was induced as described. Briefly, nursing mothers and their pups were maintained in 75%±3% oxygen from P7 to P12 and returned to room air for 5 days to P17. CNV was induced with a diode laser as described. Fundus angiography with 2% fluorescein was performed on D7 and D14 post-lasering with a scanning laser ophthalmoscope (Heidelberg Spectralis, Germany). Central avascular (AV) and neovascular (NV) areas were measured with ImageJ 1.44i (NIH, USA). Peritoneal neutrophils were isolated after intraperitoneal injection of 30ng lipopolysaccharide (LPS; Sigma, UK).

Histology
Formaldehyde-fixed tissue was labelled with biotinylated isoelectin B4 (IB4) (Life Technologies, UK) and antibodies for F4/80 (AbD Serotec, UK), ionized calcium binding adapter molecule-1 (IBA1) (Wako, Japan) or GFP/YFP (MBL International, USA), followed by fluorophore-conjugated streptavidin and appropriate secondary antibodies (Life Technologies, UK). X-gal staining and in situ hybridisation were performed on frozen eye sections.

Flow cytometry and sorting
Single-cell suspensions were stained with fluorophore-conjugated antibodies (Biolegend, UK) for CD11b (myeloid cell lineage), CD11c (dendritic cells), Ly6G (neutrophils), NK1.1 (natural killer cells), Ly6C (activation status of macrophages and monocytes). Flow cytometry data were collected on an LSRFortessa (BD Pharmingen, UK) and analysed with FlowJo (Treestar, USA). Influx sorter (BD Pharmingen, UK) was used for cell sorting.

Quantitative PCR (qPCR)
DNA and RNA were isolated using the All-prep kit (Qiagen, UK). RNA was reverse-transcribed with the QuantiTect Reverse Transcription kit (Qiagen, UK) and qPCR performed on an Applied Biosciences 7900HT thermocycler (Life Technologies, UK) using the TaqMan probe-based PerfeCTa® qPCR FastMix® (VWR, UK) or SYBR Green (Life Technologies, UK) and the following oligonucleotides: for genomic PCR, Vegfa-F 5’-ACTTCATGGACAGGCTTCGG-3’ and Vegfa-R 5’-ACATCTGCTGTGCTGTAGGAAG-3’; Hif1a-F 5’-TGATGTGGGTGCTGGTGTC-3’ and Hif1a-R 5’-TTGTGTTGGGGCAGTACTG-3’; Hif2a (Epas1)-F 5’-GAGAGCAGCTTCTCCTGGAA-3’ and Hif2a (Epas1)-R 5’-TGTAGGCAAGGAAAACCAAGG-3’; for RT-PCR, Vegfa RT-F 5’-CAGATCATGGGATCAAACT-3’ and Vegfa RT-R 5’-TTGTTCTGTTCCTGAG-3’; Hif1a RT-F 5’-TTGGAGTAAAAAACATGCTTTGGA-3’ and Hif1a RT-R 5’-GCTTCGCGGATCTTGTGC-3’; Hif2a (Epas1) RT-F 5’-CCAGGAATAACCCTCGTTT-3’ and Hif2a (Epas1) RT-R 5’-GGATTCTCTTCTCTCAATG-3’; Actb RT-F 5’-CAAGCCCTCTCAATTCGTC-3’ and Actb RT-R 5’-CCAAGGAGAATCGCTCAT-3’.

ELISA
VEGF levels were measured with the Duoset ELISA kit (R&D Systems, UK).
References:


