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▶ To cite this version:

Akiyoshi Uemura, Marcus Fruttiger, Patricia A d'Amore, Sandro de Falco, Antonia M Joussen, et al.. VEGFR1 signaling in retinal angiogenesis and microinflammation. Progress in Retinal and Eye Research, 2021, pp.100954. 10.1016/j.preteyeres.2021.100954 . hal-03155125

HAL Id: hal-03155125
https://hal.sorbonne-universite.fr/hal-03155125v1

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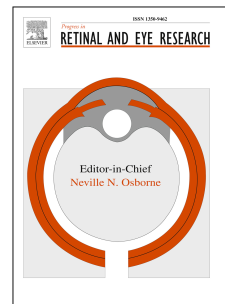

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PII: S1350-9462(21)00015-X

DOI: https://doi.org/10.1016/j.preteyeres.2021.100954

Reference: JPRR 100954

To appear in: *Progress in Retinal and Eye Research*

Received Date: 17 July 2020

Revised Date: 12 February 2021

Accepted Date: 19 February 2021

Please cite this article as: Uemura, A., Fruttiger, M., D'Amore, P.A., De Falco, S., Joussen, A.M., Sennlaub, F., Brunck, L.R., Johnson, K.T., Lambrou, G.N., Rittenhouse, K.D., Langmann, T., VEGFR1 signaling in retinal angiogenesis and microinflammation, *Progress in Retinal and Eye Research* (2021), doi: https://doi.org/10.1016/j.preteyeres.2021.100954.

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CRediT (Contributor Roles Taxonomy) Author Contributions

Manuscript: ***VEGFR1 signaling in retinal angiogenesis and microinflammation***

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Concept-ualization	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
Methodology	**Y**	**Y**	**N/A**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
Software	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**
Validation	**Y**	**Y**	**N/A**	**N/A**	**N/A**	**N/A**	**Y**	**Y**	**Y**	**N/A**	**N**
Formal analysis	**Y**	**Y**	**N/A**	**Y**	**Y**	**Y**	**Y**	**Y**	**N**	**Y**	**N**
Investigation	**Y**	**Y**	**N/A**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
Resources	**Y**	**Y**	**N/A**	**N/A**	**N/A**	**N/A**	**N**	**Y**	**Y**	**N/A**	**N**
Data Curation	**Y**	**N**	**N/A**	**N/A**	**N/A**	**N/A**	**N**	**N**	**N**	**N/A**	**N**
Writing - Original Draft	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
Writing - Review & Editing	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
Visualization	**Y**	**Y**	**N**	**Y**	**Y**	**Y**	**N**	**Y**	**Y**	**Y**	**N**
Supervision	**N**	**N**	**N/A**	**Y**	**Y**	**Y**	**Y**	**N**	**Y**	**Y**	**N**
Project administration	**N**	**Y**	**N/A**	**Y**	**Y**	**Y**	**Y**	**Y**	**N**	**Y**	**N**
Funding acquisition	**N**	**N**	**N/A**	**Y**	**Y**	**Y**	**N**	**N**	**N**	**Y**	**N**

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VEGFR1 signaling in retinal angiogenesis and microinflammation

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Author contributions

Percentage of work contributed by each author in the production of the manuscript is as follows: Akiyoshi Uemura 9%, Marcus Fruttiger 9%, Patricia A. D'Amore 9%, Sandro De Falco 9%, Antonia M. Joussen 9%, Florian Sennlaub 9%, Lynne R. Brunck 9%, Kristian T. Johnson 9%, George N. Lambrou 9%, Kay D. Rittenhouse 9%, Thomas Langmann 9%

Conflict of interest disclosures

Akiyoshi Uemura, Marcus Fruttiger and Patricia A. D'Amore declare no conflicts of interest. Sandro De Falco is co-founder of the startup AnBition s.r.l., Naples, Italy and co-inventor of the patents PCT/IB2018/057636, Peptides and medical uses thereof, Priority date 11/09/2019 and Italian patents n. 102018000008493 and n. 102018000008507, Peptidi ed usi medici correlati, priority date 11/09/2018. Antonia M. Joussen is a consultant for Allergan, Bayer, Novartis and Roche and has received research funding from Bayer and Novartis. Florian Sennlaub declares no conflict of interest. Lynne R. Brunck, Kristian T. Johnson, George N. Lambrou and Kay D. Rittenhouse are employees of Bayer Consumer Care AG. Thomas Langmann has participated in advisory boards from Bayer HealthCare AG.

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Abbreviations

AMD	Age-related macular degeneration
BRB	Blood–retina barrier
BRVO	Branch retinal vein occlusion
CCL2	CC chemokine ligand 2
CNV	Choroidal neovascularization
CRVO	Central retinal vein occlusion
CX3CR1	C-X3-C motif chemokine receptor 1
DME	Diabetic macular edema
DR	Diabetic retinopathy
EC	Endothelial cell
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
GA	Geographic atrophy
HIF	Hypoxia-inducible factor
Iba1	Ionized calcium-binding adapter molecule 1
ICAM	Intracellular adhesion molecule
IL	Interleukin
KO	Knock-out
MAPK	Mitogen-activated protein kinase
NPDR	Non-proliferative diabetic retinopathy
NRP	Neuropilin
OCT	Optical coherence tomography
OIR	Oxygen-induced retinopathy
PI3K	Phosphoinositide 3-kinase
PDGF	Platelet-derived growth factor
PDR	Proliferative diabetic retinopathy
Pgf	*Placental growth factor (mouse gene)*
PlGF	Placental growth factor (protein)
ROP	Retinopathy of prematurity
RPE	Retinal pigment epithelium
RVO	Retinal vein occlusion
sVEGFR1	Soluble/secreted VEGFR1
TGF	Transforming growth factor
TK	Tyrosine kinase
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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Abstract

Five vascular endothelial growth factor receptor (VEGFR) ligands (VEGF-A, -B, -C, -D, and placental growth factor [PlGF]) constitute the VEGF family. VEGF-A binds to VEGF receptors 1 and 2 (VEGFR1/2), whereas VEGF-B and PlGF only bind VEGFR1. Although much research has been conducted on VEGFR2 to elucidate its key role in retinal diseases, recent efforts have shown the importance and involvement of VEGFR1 and its family of ligands in angiogenesis, vascular permeability, and microinflammatory cascades within the retina. Expression of VEGFR1 depends on the microenvironment, is differentially regulated under hypoxic and inflammatory conditions, and it has been detected in retinal and choroidal endothelial cells, pericytes, retinal and choroidal mononuclear phagocytes (including microglia), Müller cells, photoreceptor cells, and the retinal pigment epithelium. Whilst the VEGF-A decoy function of VEGFR1 is well established, consequences of its direct signaling are less clear. VEGFR1 activation can affect vascular permeability and induce macrophage and microglia production of proinflammatory and proangiogenic mediators. However the ability of the VEGFR1 ligands (VEGF-A, PlGF, and VEGF-B) to compete against each other for receptor binding and to heterodimerize complicates our understanding of the relative contribution of VEGFR1 signaling alone toward the pathologic processes seen in diabetic retinopathy, retinal vascular occlusions, retinopathy of prematurity, and age-related macular degeneration. Clinically, anti-VEGF drugs have proven transformational in these pathologies and their impact on modulation of VEGFR1 signaling is still an opportunity-rich field for further research.

Keywords

Angiogenesis; Microinflammation; Placental growth factor (PlGF); Vascular endothelial growth factor-A (VEGF-A); Vascular endothelial growth factor receptor 1 (VEGFR1)

Highlights (3 to 5 bullet points, maximum 85 characters, including spaces, per bullet point)

- VEGF-A, VEGF-B, PlGF and VEGFR1/R2 can form homodimers or heterodimers
- The complexity of VEGF family ligands and receptor interactions is underappreciated
- VEGFR1 and PlGF are involved in inflammatory pathways of retinal diseases
- A contribution of VEGFR1 to retinopathy via effects on inflammation is likely
- Clinical relevance of targeting both VEGF-A/PlGF vs VEGF-A needs further elucidation

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1 **1. Introduction**

2 The retina is nourished by two distinct vascular networks: the inner retina is maintained by the
3 retinal vasculature, whereas the outer retina depends on the choroidal vasculature. The retinal
4 vasculature comprises tightly sealed endothelial cells (ECs) surrounded by pericytes and glial cells,
5 forming the inner blood–retina barrier (BRB) (Díaz-Coránguez et al., 2017; Klaassen et al., 2013). In
6 contrast, the choroidal vasculature is highly permeable, consisting of fenestrated ECs with fewer
7 pericytes, from which oxygen diffuses through the monolayered retinal pigment epithelial (RPE) cells of
8 the outer BRB to reach photoreceptor cells (Fields et al., 2020; Kur et al., 2012). At both the inner and
9 outer barriers, pathological changes can lead to uncontrolled formation of new fragile blood vessels and
10 extravasation in both the retinal and choroidal vascular beds, which can lead to severe vision
11 impairment or blindness (Klaassen et al., 2013).

12 The most common disease affecting the retinal vasculature is diabetic retinopathy (DR), the
13 prevalence of which increases with duration of diabetes (approximately 20% versus 75% in individuals
14 with diabetes for <10 versus ≥20 years) and levels of glycosylated hemoglobin (approximately 20%
15 versus 50% in individuals with levels ≤7.0 versus >9.0%), and is higher in those with type 1 versus type 2
16 (approximately 75% versus 25%) diabetes (Yau et al., 2012). Hyperglycemia in diabetes is the major long-
17 term determinant of vascular changes in DR (Klaassen et al., 2013); damage to the ECs and pericytes of
18 the inner BRB contribute to subsequent retinal edema and hemorrhage within the retina and impaired
19 vision (Kempen et al., 2004). In proliferative DR (PDR), an advanced form of DR, retinal microvascular
20 alterations lead to tissue ischemia and retinal neo-angiogenesis, which are often accompanied by
21 development of contractile fibrovascular membranes.

22 The main pathology that occurs in the outer retina is age-related macular degeneration (AMD).
23 As AMD progresses, sustained stress to RPE cells leads to a loss of photoreceptor cells, RPE cells, and the
24 underlying choriocapillaris, which in the late stage of "dry AMD" manifests as geographic atrophy (GA)
25 and leads to vision loss. In "wet AMD", choroidal neovascularization (CNV) causes a wide variety of
26 anatomical disruptions in the neural architecture of the macula, such as retinal edema and detachment
27 with hemorrhagic exudates, as well as subretinal fibrosis. Again, these changes lead to vision
28 impairment and, in more extreme cases, vision loss. Although vascular changes are the hallmark of DR
29 and AMD, there is also an increasing appreciation that within both DR and AMD there is chronic
30 microinflammation (that occurs at a cellular level in the absence of tissue injury or infection and is also
31 referred to as "subclinical" or "low-grade" inflammation (Antonelli and Kushner, 2017), "para-

32 inflammation" (Chen and Xu, 2015; Xu et al., 2009), or "inflamm-aging" (Subhi et al., 2019).

33 Vascular endothelial growth factor (VEGF) is viewed as a pivotal mediator of pathology in both
34 AMD and DR and is a target of current therapeutic interventions (Aiello, 2008). However, the VEGF
35 signaling pathway is complex and includes multiple ligand–receptor interactions that regulate diverse
36 processes in different cell types, in a context-dependent manner (Penn et al., 2008). To date, the best
37 characterized process is activation of VEGF receptor 2 (VEGFR2) tyrosine kinase (TK) in ECs by VEGF-A,
38 which induces angiogenesis and increases vascular permeability (Peach et al., 2018). In contrast, VEGF
39 receptor 1 (VEGFR1) functions, in part, as a decoy for VEGF-A, attenuating VEGFR2/VEGF-A–mediated
40 outcomes (Koch and Claesson-Welsh, 2012). In addition, an established body of evidence indicates
41 disease-specific roles of direct VEGFR1 signaling, which are independent of VEGFR1 decoy activity, in
42 particular in immune cells expressing VEGFR1 (Clauss et al., 1996; Crespo-Garcia et al., 2017; Huang et
43 al., 2013; Luttun et al., 2002; Muramatsu et al., 2010). Yet the complexities in the ligand–receptor
44 interactions and their differential expression have so far precluded a clear understanding of VEGFR1
45 functions.

46 In this review, we summarize the current understanding of the signals mediated by VEGFR1 and
47 its ligands – VEGF-A, VEGF-B, and placental growth factor (PlGF) – within the retinal microenvironment
48 in both healthy and pathologic states. We further discuss the possibility of translating the knowledge
49 gained from basic science into the clinical management of DR and AMD, as well as other eye diseases.
50 The goal of this review is to highlight the key research in this area and guide future research in this
51 constantly evolving field. We recognize that this area of research is extensive, spanning pharmacology to
52 animal models to clinical trials and it is clear that the science of VEGFR1 continues to grow, as evidenced
53 by the consistently increasing number of publications on this topic. Accordingly, the review begins by
54 introducing the current biology of the VEGFR1 itself and in context with VEGFR2, followed by an
55 overview of disease-driven biological processes where VEGFR1 is known to have a role, focusing on
56 retinal vascular and macular degenerative diseases.

57 2. Molecular signaling mechanisms in the VEGF family

58 *2.1. Basic VEGF receptor properties*

59 There are three evolutionarily related VEGF receptors in humans: VEGFR1 (FLT1), VEGFR2
60 (KDR/FLK1), and VEGFR3 (FLT4) (Grassot et al., 2006). They all comprise an extracellular, ligand-binding

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61 domain composed of seven immunoglobulin-like loops, a transmembrane domain, a juxtamembrane
62 domain, a split intracellular TK domain, and a C-terminal tail; all three have different ligand-binding
63 properties and biological functions (D'Amore, 1994; De Falco, 2012; de Vries et al., 1992; Ebos et al.,
64 2004; Jeltsch et al., 2013; Shibuya, 2013; Takahashi and Shibuya, 1997; Terman et al., 1992; Vaisman et
65 al., 1990).

66 There are five VEGFR ligands (VEGF-A, -B, -C, -D, and PlGF) collectively known as the VEGF
67 family. Isolated and cloned in 1989, VEGF-A was confirmed as a disulfide-linked dimeric glycoprotein
68 with EC growth-promoting properties (Ferrara, 2011; Jakeman et al., 1992; Keck et al., 1989; Leung et
69 al., 1989). Of all the VEGF family members, PlGF shares the greatest homology with VEGF-A, despite its
70 unique nomenclature, which derives from the fact that it was initially isolated from a human placental
71 complementary DNA library (Iyer et al., 2001). The VEGF-A mRNA contains eight exons, the splicing of
72 which gives rise to a variety of isoforms. To date, 16 distinct VEGFA isoforms have been identified most
73 commonly from six transcripts: $VEGF_{111}$, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$; the subscripted
74 numbers denoting the number of amino acids present (Peach et al., 2018). The isoforms have different
75 affinities for extracellular matrix components, VEGF receptors and associated coreceptors, and the
76 expression profile of each isoform varies among tissues (Ng et al., 2001). One of the primary differences
77 among the VEGF-A isoforms is their ability to bind heparan sulfate proteoglycan, affecting their
78 diffusiblilty within tissues: the larger isoforms can bind heparan sulfate proteoglycan, whereas VEGF-
79 A120 in mice and VEGF-A121 in humans do not (Bridgett et al., 2017; Stalmans et al., 2002). Alternative
80 splicing of the VEGF-A pre-mRNA may also give rise to a non- or anti-angiogenic family of isoforms
81 ($VEGF\text{-}A_{xxxb}$) as well as the pro-angiogenic family ($VEGF\text{-}A_{xxxa}$) (Bowler and Oltean, 2019; Peach et al.,
82 2018). However, the relevance of these transcripts has been contested (Bridgett et al., 2017; Harris et
83 al., 2012; Lomet et al., 2018). Furthermore, alternative splicing also gives rise to multiple isoforms of
84 PlGF and VEGF-B (Olofsson et al., 1996; Yang et al., 2003).

85 *2.2. Modulation of VEGFR1/2 signaling*

86 VEGF-A binds to VEGFR1 and VEGFR2, whereas VEGF-B and PlGF only bind VEGFR1, and VEGF-C
87 and VEGF-D primarily bind VEGFR3 (Jeltsch et al., 2013), although proteolytically processed VEGF-C and
88 VEGF-D can also bind to VEGFR2 (Achen et al., 1998; Joukov et al., 1996; Joukov et al., 1997; Masoumi
89 Moghaddam et al., 2012; Stacker et al., 1999). However, as the VEGF-C/VEGF-D/VEGFR3 pathway mainly
90 regulates the formation of lymphatic vessels, which are absent in the retina (Alitalo, 2002), this review

91 focuses primarily on VEGFR1 and its ligands (VEGF-A, VEGF-B, and PlGF), and delineates the roles
92 between the VEGFR1 and VEGFR2 receptors in retinal development, homeostasis, and disease.

93 VEGFR1/2 activation and signaling are heavily influenced by a number of elements, and a key
94 one is the availability of the VEGFR ligands themselves, which are modulated by a large array of
95 mechanisms. First, the availability of VEGFR ligands depends on transcriptional and post-transcriptional
96 regulation of their expression. Second, specific ligands can bind to and activate VEGFR1, causing VEGFR1
97 to signal via its own kinase domain, but VEGFR1 also has indirect effects on VEGFR2 activity by acting as
98 a decoy receptor for VEGFR2 ligands (Koch and Claesson-Welsh, 2012). Third, VEGF-A, VEGF-B, and PlGF
99 can compete for VEGFRs and extracellular matrix binding sites (Koch and Claesson-Welsh, 2012). Fourth,
100 VEGF-A, VEGF-B, and PlGF can form heterodimers and VEGFR subtypes can form homodimers or
101 heterodimers, depending on the binding ligand (Koch and Claesson-Welsh, 2012). Fifth, VEGFR1/2
102 activity can also be modulated by neuropilins and the glycocalyx component, endomucin, which act as
103 VEGF co-receptors (Alvarez-Aznar et al., 2017; LeBlanc et al., 2019).

104 *2.2.1. Expression of VEGFR1 ligands and VEGFRs*

105 VEGFR1 expression has been detected in various types of cells, including retinal and choroidal
106 ECs (Cao et al., 2010; Fruttiger, 2002; Stewart et al., 2011), retinal pericytes (Cao et al., 2010; Eilken et
107 al., 2017), retinal and choroidal mononuclear phagocytes (Couturier et al., 2014; Huang et al., 2013;
108 Ogura et al., 2017), Müller cells (Stitt et al., 1998), photoreceptor cells (Luo et al., 2013), and RPE cells
109 (Luo et al., 2013) as shown in **Fig. 1a**. It should be noted that the VEGFR1 expression in these cell types is
110 variable depending on their microenvironments in developmental, homeostatic, and disease conditions.
111 In particular, oxygen concentration and inflammation largely influence the expression levels of VEGFR1
112 as well as its ligands (Hata et al., 1995; Philipp et al., 2000).

113 Michaelson et al. first introduced the concept that damage to the ECs and/or pericytes of the
114 vascular network may lead to vessel closure and ultimately, hypoxia within the retina (Michaelson et al.,
115 1954). Soon afterwards, Wise proposed the presence of a “hypoxia-induced growth factor” (Wise, 1961),
116 which was identified as VEGF-A over the subsequent decades. Mechanistically, it has been shown that
117 tissue exposure to hypoxia provokes an adaptive response that is reliant on the ability of retinal cells to
118 detect alterations in intracellular oxygen tension.

119 Hypoxia-induced expression of VEGF-A can be mediated by both transcriptional and post-
120 transcriptional mechanisms. The transcriptional response depends on hypoxia-inducible factor (HIF)-1

121 and HIF-2, heterodimeric transcription factors that modulate the expression of a large set of genes
122 through binding to the hypoxia responsive element located in the promoters or other genomic
123 regulatory regions (Forsythe et al., 1996; Gerber et al., 1997; Maxwell and Ratcliffe, 2002; Semenza,
124 2001). VEGF-A mRNA is intrinsically labile and contains destabilizing elements in its 5', 3' untranslated,
125 and coding regions (Dibbens et al., 1999; Levy, 1998; Yao et al., 2013). Hypoxic conditions stablize VEGF-
126 A mRNA (Dibbens et al., 1999; Levy, 1998; Yao et al., 2013), increasing its half-life from about 45 minutes
127 to more than 8 hours (Shima et al., 1995). This post-transcriptional mechanism can have a greater
128 impact on VEGF-A protein production than transcriptional regulation.

129 HIF-mediated gene regulation also impacts expression of other members of the VEGF family and
130 related receptors. For instance, HIF-1 activates transcription of VEGFR1, but not VEGFR2 (Gerber et al.,
131 1997). More recently, the molecular mechanisms underlying the positive modulation of PlGF expression
132 by hypoxia in vascular cells at transcriptional (Tudisco et al., 2017) and post-transcriptional (Xiang et al.,
133 2014) levels have been unraveled. Furthermore, hypoxia-related modulation of PlGF expression has
134 been shown to be mediated by metal-responsive transcription factor-1 in immortalized/H-Ras–
135 transformed mouse embryonic fibroblasts (Green et al., 2001), and by nuclear factor-κB in human
136 embryonic kidney 293 cells (Cramer et al., 2005).

137 Several other mechanisms regulate gene expression in the VEGF family. For instance, VEGF-A
138 expression is upregulated by several growth factors, including epidermal growth factor, transforming
139 growth factors (TGF) α and β, insulin-like growth factor-1, fibroblast growth factor, and platelet-derived
140 growth factor (PDGF) (Ferrara et al., 2003). Inflammatory cytokines, such as interleukin (IL)-1β and IL-6,
141 stimulate expression of VEGF-A in several cell types, modulating angiogenesis and vascular permeability
142 in inflammatory conditions (Ferrara et al., 2003). Oncogenic mutations can also influence VEGF-A
143 expression (Ferrara et al., 2003). Similarly, activation of oncogenes (Ras), inflammatory cytokines (IL-1β
144 and tumor necrosis factor [TNF]-α), and several growth factors can also positively modulate PlGF
145 expression in pathologic conditions, including many types of cancer and chronic inflammatory conditions
146 (Kim et al., 2012).

147 *2.2.2. Decoy function of VEGFR1*

148 Animal studies provided evidence of the essential function of VEGFR1 in vascular development
149 as mice lacking VEGFR1 die at embryonic day 8.5 due to an excess of ECs, which assemble into
150 disorganized tubules (Fong et al., 1995). In contrast, mice engineered to express a truncated, non-

151 signaling form of VEGFR1 lacking the TK domain (*VEGFR1-TK-/-*) (Hiratsuka et al., 1998) are healthy and
152 fertile with close to normal vascularization. Similarly, knock-out (KO) mice for genes encoding the
153 VEGFR1-specific ligands, PlGF (Apicella et al., 2018; Carmeliet et al., 2001) and VEGF-B (Bellomo et al.,
154 2000), are also largely normal.

155 Although VEGF-A binds to VEGFR1 with higher affinity than VEGFR2 (K_d =15 pM vs 750 pM),
156 VEGFR1 exhibits 10-times lower TK activity (Sawano et al., 2001; Shinkai et al., 1998). Based on initial
157 observations, it appears that one role of VEGFR1 is to act as a decoy receptor for VEGF-A rather than a
158 signaling mediator, which limits the activity of the VEGF-A/VEGFR2 axis, at least in physiological settings
159 (Carmeliet et al., 2001). In addition, a soluble/secreted version of VEGFR1 (sVEGFR1) can be produced
160 via alternative splicing or proteolytic cleavage retaining the extracellular ligand–binding domains of
161 VEGFR1 (Kendall and Thomas, 1993; Raikwar et al., 2013), lowering the availability of free VEGF-A in the
162 extracellular space and indirectly modulating the intensity of VEGFR2 signaling (Kappas et al., 2008). The
163 powerful anti-VEGF-A activity of sVEGFR1 is seen in the cornea, where it is strongly expressed and plays
164 a crucial role in maintaining corneal avascularity (Ambati et al., 2006).

165 *2.2.3. Competition among VEGFR1 ligands*

166 Because VEGFR1 functions as a decoy receptor for VEGF-A, the other two VEGFR1 ligands, PlGF
167 and VEGF-B, can indirectly affect VEGF-A availability by competing for VEGFR1 binding. For example,
168 increased levels of VEGF-B can increase unbound VEGF-A levels by preventing VEGF-A from being
169 trapped by VEGFR1, indirectly leading to increased VEGFR2 activation (Anisimov et al., 2013; Kivela et
170 al., 2014; Robciuc et al., 2016). In other words, despite its lack of affinity for VEGFR2, VEGF-B can still
171 indirectly activate VEGFR2 signaling, if VEGF-A is present.

172 Likewise, PlGF can also increase unbound VEGF-A levels by competing for VEGFR1 binding (Yang
173 et al., 2013) and displacing VEGF-A from VEGFR1 (Carmeliet et al., 2001; Kowalczuk et al., 2011; Park et
174 al., 1994). However, competition between PlGF and VEGF-A for VEGFR1 binding is complicated by PlGF
175 also being capable of directly activating VEGFR1 signaling. Indeed, PlGF can directly stimulate vessel
176 growth by inducing proliferation, migration, and survival of ECs (Adini et al., 2002; Carmeliet et al., 2001;
177 Ziche et al., 1997a), as well as vessel maturation, by increasing the proliferation and recruitment of
178 vascular smooth muscle cells (Bellik et al., 2005; Yonekura et al., 1999). Thus, when interpreting
179 biological outcomes, it is important to consider the possibility of effects of PlGF and VEGF-B acting
180 directly via VEGFR1, or indirectly via VEGFR2 due to VEGF-A displacement.

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181 In summary, PlGF and VEGF-B are able to compete with VEGF-A in binding to VEGFR1, freeing up
182 VEGF-A. The relative binding affinities of VEGF-A and PlGF to VEGFR1 have been assessed under various
183 conditions, including levels of glycosylation of the ligands and binding to specific domains of the
184 receptor (Huang et al., 2019a; Jiao et al., 2019). To date, it remains unclear whether VEGF-A or PlGF
185 binds more tightly to VEGFR1.

186 *2.2.4. Heterodimerization of VEGFR1 ligands and VEGFRs*

187 VEGF-A, VEGF-B, and PlGF can form heterodimers (**Fig. 2**) if they are co-expressed in the same
188 cell (Cao et al., 1996; DiSalvo et al., 1995). The formation of VEGF-A/PlGF heterodimers can reduce the
189 number of VEGF-A homodimers formed, thereby reducing signaling via VEGFR2. On the other hand,
190 VEGF-A/PlGF heterodimers can still bind VEGFR1, competing with VEGF-A and increasing the amount of
191 unbound VEGF-A available for VEGFR2 binding (Autiero et al., 2003; Tarallo et al., 2010; Yang et al.,
192 2013). It is therefore extremely difficult to predict the effects of PlGF because it can either increase
193 VEGFR2 signaling (by freeing up VEGF-A from VEGFR1) or decrease VEGFR2 signaling (by trapping VEGF-
194 A in VEGF-A/PLGF heterodimers).What mechanism dominates under which circumstances is currently
195 not known, but the complexity of the system may be responsible for some of the apparent contradictory
196 in vivo findings about PlGF described below in section 3.

197 To complicate matters further, VEGF-A/PlGF heterodimers, like VEGF-A, can bind VEGFR1/2
198 heterodimers (Autiero et al., 2003). In fact, VEGFR1/2 heterodimers exist even in the absence of VEGF-
199 A/PlGF heterodimer ligands (**Fig. 2**) (Autiero et al., 2003). Computational modeling has shown that, in
200 cells expressing both receptors, VEGFR1/2 heterodimers comprise 10–50% of active, signaling VEGFR
201 complexes, and form preferentially over VEGFR1 homodimers when VEGFR2 is more abundant (Mac
202 Gabhann and Popel, 2007). It has been suggested that VEGFR1/2 heterodimers reduce signaling via
203 VEGFR2 homodimers (Cai et al., 2017; Cudmore et al., 2012), but the signal transduction properties of
204 VEGFR heterodimers are currently not well characterized and their functional roles in an *in vivo* context
205 are, at this stage, very difficult to predict. Nevertheless, some of the known biological effects of VEGF-
206 A/PlGF and VEGFR1/2 heterdimers are discussed further in sections 3.1.2 and 3.2.

207 *2.2.5. VEGF co-receptors*

208 Neuropilin (NRP)-1 and -2 were first identified as co-receptors for semaphorin and VEGF
209 signaling during neural and vascular development (Giger et al., 1998; Gu et al., 2003; Sulpice et al.,

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210 2008). NRPs are transmembrane proteins with a small cytoplasmic domain that lack intrinsic catalytic
211 function (Fujisawa et al., 1997). The larger heparin-binding members of the three VEGF ligands (VEGF-A,
212 VEGF-B, and PlGF) are able to bind NRP1 and NRP2 (Makinen et al., 1999; Migdal et al., 1998), bridging
213 VEGFRs and NRP1 or NRP2 to create holoreceptor complexes (Pellet-Many et al., 2008; Wild et al., 2012)
214 and inducing intracellular trafficking of VEGFR2, which is a critical event for downstream signal
215 transduction (Simons et al., 2016). In ECs, NRPs modulate VEGFR signaling, enhancing migration (Soker
216 et al., 1998) and survival (Favier et al., 2006). In addition, NRP-1 has also been implicated in the spatial
217 organization of ECs within angiogenic sprouts (Fantin et al., 2013; Kawamura et al., 2008) and in mouse
218 models of pathological choroidal and retinal neovascularization (Dejda et al., 2014; Dejda et al., 2016;
219 Fernandez-Robredo et al., 2017).

220 The biological relevance of NRP-1 for VEGF signaling has yet to be fully elucidated because mice
221 with a mutant version of NRP-1 that cannot bind VEGF develop normally (Gelfand et al., 2014).
222 Furthermore, NRPs have been shown to bind other growth factors, such as TGF-β (Glinka and
223 Prud'homme, 2008), fibroblast growth factor, and others (Uniewicz and Fernig, 2008; West et al., 2005).
224 In ECs, NRP-1 plays an important role during sprouting angiogenesis, modulating differential
225 responsiveness to TGF-β superfamily signaling, independently of VEGF-A (Aspalter et al., 2015). The
226 relevance of interactions between NRPs and other growth factors in the context of vascular biology
227 remains to be established.

228 More recently, biochemical studies have revealed that the glycocalyx component endomucin
229 interacts with VEGFR2 (independent of the presence of VEGF-A) and that knock-down of endomucin in
230 cultured human retinal ECs using small interfering RNA blocks the biologic action of VEGF-A by
231 preventing VEGFR2 internalization (LeBlanc et al., 2019; Park-Windhol et al., 2017). Preliminary studies
232 indicate that VEGFR1 internalization also requires the presence of endomucin, but it is unclear if this
233 applies to VEGFR1 homodimers or requires dimerization between VEGFR1 and VEGFR2.

234 *2.3. Downstream signaling of VEGFRs*

235 Upon ligand binding, conformational changes in the VEGFR intracellular domains lead to
236 autophosphorylation of specific tyrosine residues. This allows binding of several signaling mediators
237 such as phospholipase C gamma, non-receptor TKs such as Src, and adaptor proteins, such as those
238 containing the Src homology 2 domain. Consequently, VEGFR1 and VEGFR2 activation induce signaling
239 pathways that are normally activated by TK receptors (**Fig. 1b**), such as extracellular signal-regulated

240 kinase (ERK)/mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and p38
241 (Jeltsch et al., 2013; Koch and Claesson-Welsh, 2012). Downstream signals from these receptors
242 converge to cooperatively regulate transcription of different genes, leading to cell proliferation,
243 migration, and survival, as well as controlling cell–cell contacts, cell–matrix adhesions, and cytoskeletal
244 rearrangements (**Fig. 1c**), depending on cell type and biological context (Jeltsch et al., 2013; Koch and
245 Claesson-Welsh, 2012).

246 To date, there is little comparative evidence regarding the relative activation of the different
247 VEGFR1 signaling pathways following binding by VEGF-A and PlGF homodimers and heterodimers.
248 However, there are important differences between the downstream signaling mechanisms induced by
249 activation of VEGFR1 and VEGFR2. VEGFR2 has strong TK activity that induces a plethora of signals
250 depending on which tyrosine becomes phosphorylated after ligand binding, whereas VEGFR1 has
251 comparatively weak kinase activity (Koch and Claesson-Welsh, 2012; Meyer et al., 2006; Olsson et al.,
252 2006), although some studies suggest potentially stronger downstream activity when PlGF binds VEGFR1
253 compared to VEGF-A (Roskoski, 2008). Like many other TK receptors, VEGFRs are internalized by
254 clathrin-mediated endocytosis upon ligand binding and are subsequently proteolytically degraded
255 (Pitulescu and Adams, 2014).

256 The downstream signaling of VEGFRs can be modified by VEGFR interactions. Specifically, PlGF is
257 known to regulate intermolecular and intramolecular cross talk between VEGFR1 and VEGFR2. For
258 example, VEGFR1 activated by PlGF can trans-phosphorylate VEGFR2 (Autiero et al., 2003). Furthermore,
259 signaling via VEGFR1/2 heterodimers can lead to outcomes that are different to VEGFR2 homodimer
260 signaling (Cudmore et al., 2012). Activation of VEGFR1 by VEGF-A and PlGF homodimers, as well as by
261 VEGF-A/PlGF heterodimers, induces overlapping pathways, but also a distinct downstream response.
262 The pattern of VEGFR1 tyrosine phosphorylation differs in a ligand-dependent manner. PlGF, but not
263 VEGF-A, directly stimulates ECs through the phosphorylation of tyrosine residues 1213 (Autiero et al.,
264 2003) and 1309 (Dewerchin and Carmeliet, 2012; Fischer et al., 2008; Koch and Claesson-Welsh, 2012;
265 Selvaraj et al., 2003). VEGFR1 phosphorylation is stimulated by VEGF-A but fails to alter the gene
266 expression profile of mouse capillary ECs, whereas PlGF stimulation induces the expression of more than
267 50 genes (Roskoski, 2008). Furthermore, VEGF-A/PlGF and VEGF-A, but not PlGF, induce Akt-mediated
268 cyclin-dependent kinase inhibitor 1B (p27Kip1) phosphorylation at residue Thr198 that is associated
269 with its cytoplasmic retention and stimulation of cell motility (Apicella et al., 2018). Use of a synthetic
270 heterodimer that does not occur naturally in humans, comprising VEGF-E (a non-mammalian protein)

271 and PlGF, which specifically bind VEGFR2 and VEGFR1, respectively, activates the VEGFR1/2 heterodimer
272 and highlights the ability of a receptor heterodimer to regulate EC homeostasis, migration, and
273 vasorelaxation via the nitric oxide pathway (Cudmore et al., 2012).

274 It is clear from the research to date that the relationship between PlGF and VEGF-A and their
275 interactions with the VEGFR1 and VEGFR2 receptors is remarkably complex and the resulting
276 downstream effects are multifaceted. What is much less clear are the factors that determine which
277 downstream signaling pathways are activated/modulated, and how these translate into functional
278 responses, depending on the circumstances. However, the recent emergence of single cell analysis is
279 likely to facilitate progress in this field in the near future.

280 3. Biological functions of VEGFR1

281 Activation of the signaling cascades downstream of VEGFRs can lead to numerous biological
282 outcomes, depending on cell type and the expression profiles of VEGFRs/ligands in the tissue, as well as
283 the presence of other growth factors and cytokines. Therefore, to understand the biological function of
284 VEGFR1, it is important to consider all the signal-modulating mechanisms outlined in section 2.

285 *3.1. Angiogenesis*

286 VEGF-A is the prototypical VEGF family member and stimulates angiogenesis via VEGFR2
287 activation in ECs in both physiological and pathological settings (Cebe-Suarez et al., 2006; Ferrara, 2009;
288 Jakeman et al., 1992; Koch and Claesson-Welsh, 2012; Nagy et al., 2007). Indeed, homozygous VEGFR2
289 KO mice die at embryonic day 8.5 due to the defective cardiovascular development (Shalaby et al.,
290 1995). Importantly, even single allelic VEGF-A deficiency results in embryonic lethality (Carmeliet et al.,
291 1996; Ferrara et al., 1996), indicating that VEGF-A–mediated vascular formation is strictly dose-
292 dependent. During retinal development, oxygen demand in differentiating neurons induces VEGF-A
293 expression in astrocytes and Müller glia, which stimulates sprouting and lumenization of new blood
294 vessels, leading to the formation of superficial and deep layers of the retinal vasculature (Blanco and
295 Gerhardt, 2013; Claxton and Fruttiger, 2003; Liu et al., 2006; Pierce et al., 1995; Selvam et al., 2018;
296 Stone et al., 1995; Zhang et al., 2007). Likewise, VEGF-A signaling is also fundamental for retinal,
297 subretinal, and choroidal neovascularization in various eye diseases (Campochiaro, 2015).

298 *3.1.1. VEGFR1 decoy activity during sprouting angiogenesis*

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299 Endothelial cells in angiogenic sprouts display distinct phenotypes depending on their position.
300 At the front of the sprout, tip cells “sense” their environment using long filopodia, essentially navigating
301 along a VEGF-A gradient (Gerhardt and Betsholtz, 2005; Gerhardt et al., 2003; Jeltsch et al., 2013). VEGF-
302 A increases delta-like 4 expression in tip cells, which in turn induces a stalk cell phenotype in more
303 proximal ECs via Notch-mediated lateral inhibition (Blanco and Gerhardt, 2013; Hellstrom et al., 2007;
304 Jakobsson et al., 2009), triggering downregulation of VEGFR2 and upregulation of VEGFR1 in stalk cells.
305 Accordingly, sequestration of VEGF-A by VEGFR1 on these stalk cells results in spatial restriction and fine
306 tuning of VEGF-A signaling at the growing vascular front.

307 Pericytes associated with angiogenic sprouts also express transmembrane and soluble VEGFR1,
308 and the genetic ablation or the biochemical inhibition of PlGF or VEGFR1 in tumor models have
309 implicated a direct role of VEGFR1 signaling in pericyte recruitment and vessel stabilization (Cicatiello et
310 al., 2015; Tarallo et al., 2010). On the other hand, the developing retinal vasculature of the pericyte-
311 specific VEGFR1 KO mice displayed normal numbers of pericytes but increased numbers of ECs and
312 angiogenic sprouts with abnormally expanded morphology, suggesting that VEGFR1 on pericytes
313 spatially restricts VEGF signaling at the angiogenic sprout (Eilken et al., 2017). Thus, the main role of
314 VEGFR1 on pericytes awaits further investigation.

315 *3.1.2. Activity of VEGFR1 ligands in sprouting angiogenesis*

316 The role of direct VEGFR1 mediated signaling is not obvious, because (1) *VEGFR1-TK-/-* mice
317 survive and do not develop any obviously detrimental phenotypes (Hiratsuka et al., 1998); (2) *Pgf* KO
318 mice display only very subtle developmental angiogenic abnormalities, with small and transient
319 reductions in angiogenic sprouting during retinal and brain vascularization (Kay et al., 2017; Luna et al.,
320 2016), and (3) VEGF-B KO mice also appear to have a largely normal phenotype (Bellomo et al., 2000).
321 Nevertheless, the most pronounced effects of disrupting the VEGFR1 signaling axis have been observed
322 in the context of pathologies. Tumor growth (Apicella et al., 2018; Carmeliet et al., 2001), arthritis (Yoo
323 et al., 2009), and recovery from heart (Luttun et al., 2002; Pipp et al., 2003) and limb ischemia (Gigante
324 et al., 2006) were all reduced in the absence of PlGF-mediated signaling. Furthermore, angiogenesis in
325 ischemic retinas and laser-injured choroids, as well as diabetes-induced retinal cell death, capillary
326 degeneration, pericyte loss, and BRB breakdown were alleviated in *Pgf* KO mice or by pharmacologically
327 inhibiting PlGF activity (Apicella et al., 2018; Carmeliet et al., 2001; Crespo-Garcia et al., 2017; Huang et
328 al., 2015; Rakic et al., 2003).

329 In general, artificially increasing levels of VEGFR1 ligands can result in more obvious phenotypes,
330 usually characterized by increased angiogenesis. For instance, transgenic mice overexpressing PlGF in
331 the skin under the keratin-14 promoter have a substantial increase in the number and size of dermal
332 blood vessels (Odorisio et al., 2002). Similarly, adenovirus-mediated *Pgf* transfer in ischemic heart and
333 limb tissue elicits a strong angiogenic response that is comparable to that of VEGF-A (Luttun et al.,
334 2002). Transcranial injection of adeno-associated virus vectors encoding Pgf induced a robust
335 stimulation of angiogenesis and arteriogenesis in the central nervous system (Gaal et al., 2013).

336 However, findings have not been entirely consistent. For example, one study has indicated that
337 reduction of VEGF-B activity (using VEGF-B–KO mice or an anti-VEGF-B antibody) and may improve
338 diabetic readouts in mice (Hagberg et al., 2012), whereas another report suggested that diabetic disease
339 hallmarks can be improved by increasing VEGF-B, rather than blocking its activity (Robciuc et al., 2016).
340 Transgenic overexpression of PlGF in T cells under the CD2 promoter produced a significant reduction in
341 placental angiogenesis that was linked to the inhibition of BRAF and activation of ERK, (Kang et al.,
342 2014) indicating that the effects of PlGF on angiogenesis are context dependent. Moreover, reduced
343 angiogenesis has been described after blocking PlGF in mouse tumor models (Van de Veire et al., 2010),
344 but this was not confirmed by others (Bais et al., 2010).

345 Such conflicting results could arise from the complexity and context-dependency of VEGFR1
346 signaling and, as detailed in section 2, there are several molecular mechanisms that can lead to different
347 outcomes. For example, increased PlGF production can increase VEGF-A activity and angiogenesis via
348 competition for VEGFR1 binding, whereas, if PlGF is expressed in the same cell, formation of PlGF/VEGF-
349 A heterodimers could result in reduced VEGFR2 homodimer activation. In fact, co-expression of VEGF-A
350 and PlGF occurs in many cell types, including ECs and pericytes (Yonekura et al., 1999), fibroblasts
351 (Green et al., 2001), macrophages (Bottomley et al., 2000), keratinocytes (Failla et al., 2000), and RPE
352 cells (Klettner et al., 2015), making it difficult to delineate which biological activity is attributable to
353 VEGF-A or PlGF homodimers versus PlGF/VEGF-A heterodimers (DiSalvo et al., 1995).

354 Recent work by Apicella et al. suggests that PlGF/VEGF-A heterodimers do have a positive effect
355 on angiogenesis (Apicella et al., 2018), which may be mediated by a VEGFR1/2 heterodimer (Yang et al.,
356 2013). The Pgf-DE knock-in mouse, generated by knocking into the *Pgf* locus a variant (Pgf-DE) that is
357 unable to bind and activate VEGFR1 (Tarallo et al., 2010), allowed the investigation of the effects of the
358 complete loss of function of PlGF, as these mice produce homodimers of PlGF-DE and heterodimers of
359 VEGF-A/PlGF-DE that are inactive (Apicella et al., 2018). These mice showed significant impairment of

360 angiogenesis in tumor growth, hind limb ischemia, and CNV compared with *Pgf* KO and wild-type mice
361 (**Fig. 3**). Moreover, in a laser-induced CNV model, these mice show a large reduction in vascular leakage.
362 In parallel, the recombinant VEGF-A/PlGF heterodimer is able to rescue vascularization and vascular
363 leakage to an extent that is similar to that of recombinant VEGF-A (Apicella et al., 2018). These results
364 highlight the central role of the VEGF-A/PlGF heterodimer on vascular leakage and neo-angiogenesis
365 stimulation during CNV.

366 *3.2. Vascular permeability*

367 Optimal functioning of the neuronal cells of the retina necessitates a tightly regulated
368 environment in each of the functional compartments. In the healthy state, this is achieved through an
369 intact BRB that provides such an environment, and, through the cellular barriers, allows the uptake of
370 essential nutrients and elimination of discarded metabolites. As stated in the introduction, there are two
371 distinct barriers protecting the retina. The outer BRB, mediated by the RPE, regulates transport between
372 the choriocapillaris and the outer retina (Fields et al., 2020). The inner BRB regulates transport across
373 the retinal capillaries within the inner retina and is composed of a single layer of tightly adherent
374 endothelial cells, a basal lamina, and surrounding pericytes, astrocytes, and microglia (Díaz-Coránguez et
375 al., 2017; Klaassen et al., 2013). In retinal capillaries, pericytes contribute to maintenance of barrier
376 function, not only by providing mechanical support, but also by communicating with ECs via paracrine
377 signals and direct cell–cell contact (Armulik et al., 2011).

378 Vascular permeability is thought to be mediated via two mechanisms: the transcellular route
379 and the paracellular route. The transcellular route involves vesicular transport and the formation of
380 channels from vesicles or vacuoles, the vesiculo-vacuolar organelle. The paracellular route is based on
381 transient changes in junctions between ECs (adherens and tight junctions) (Wettschureck et al., 2019).
382 VEGF-A was initially identified as a factor secreted by tumor cells that induces vascular permeability
383 (Senger et al., 1983). The process is mediated via VEGFR2 and is likely to involve the tyrosine
384 phosphorylation, internalization, and degradation of vascular endothelial-cadherin, a major component
385 of endothelial adherens junctions (Dejana, 2004; Dejana and Orsenigo, 2013; Dejana et al., 2008). It may
386 also be attributable to transcellular extravasation via EC vesicles (Lin et al., 2007). VEGF-A/VEGFR2-
387 mediated modulation of vascular permeability and plasma extravasation also involves the activation of
388 endothelial nitric oxide synthase (eNOS) and production of nitric oxide (Papapetropoulos et al., 1997;
389 Ziche et al., 1997b).

390 Despite the fact that vascular permeability is generally thought to be mediated primarily by
391 VEGF-A/VEGFR2, there is also evidence for a role for VEGFR1, PlGF, and VEGF-A/PlGF heterodimers.
392 VEGFR1 activation controls vascular permeability via eNOS activation, as is the case for VEGFR2. In
393 addition, there is a functional link between PlGF and eNOS activation. The phosphorylation of eNOS,
394 which occurs downstream of ERK and Akt activation (Feliers et al., 2005; Hisamoto et al., 2001), has
395 been directly associated with the activation of VEGFR1 (Bussolati et al., 2001), and is supported by
396 findings that vascular leakage in mouse models can be reduced by blocking PlGF (Carmeliet et al., 2001)
397 or VEGFR1 (Huang et al., 2011). An in vitro study of the mechanism of high-glucose-induced damage to
398 retinal ECs concluded that PlGF was signaling through the Erk1/2-NOS axis via VEGFR1 (Jiao et al., 2019).
399 A recent study has reported that PlGF directly disrupts barrier function by suppression of glucose-6-
400 phosphate dehydrogenase and peroxiredoxin, acting through glutathione peroxidase and
401 phospholipase A_2 activity (Huang et al., 2019a). VEGF-A also induces vasodilation, mainly through
402 VEGFR1 signaling, with the involvement of VEGFR1/2 receptor heterodimerization (Cudmore et al.,
403 2012), a finding that has been corroborated using the Pgf-DE knock-in mouse model (Apicella et al.,
404 2018) described in section 3.1.2.

405 In ECs, VEGFR1 and VEGFR2 are distributed luminally and abluminally, respectively suggesting
406 that the highly polarized signaling depends on the receptor position (Hudson et al., 2014). The majority
407 of VEGFR1 is localized to the apical or luminal sides of retinal microvascular ECs, whereas VEGFR2 is
408 predominantly located on the basal or abluminal sides (Hudson et al., 2014); however, this
409 generalization has been the subject of recent debate (Blaauwgeers et al., 1999; Dragoni and Turowski,
410 2018; Van Bergen et al., 2019). Thus, luminal and abluminal endothelial surfaces display differential
411 functionality, with luminal VEGFR1 activation via circulating VEGF-A leading to Akt activation and
412 facilitation of EC survival and abluminal VEGFR2 activation via tissue-borne VEGF-A associated with
413 increased permeability via p38 (Hudson et al., 2014). This suggests important roles for the MAPK and
414 PI3K/Akt pathways downstream of VEGFR2 in angiogenesis and permeability. The impact of differential
415 apicobasal signaling, as a consequence of VEGFR1 or VEGFR2 activation, should be considered when
416 working with experimental models of retinal disease (Cao et al., 2010; Liu et al., 2017).

417 *3.3. VEGFR1 and inflammation*

418 Although not necessarily the primary etiopathogenic factor, persistent microinflammation can
419 cause considerable collateral damage in many age-related chronic diseases, fueling further inflammation

420 (Nathan and Ding, 2010) and is a known contributor to retinal pathology as well (Rashid et al., 2019). In
421 the affected tissues, inflammation is often associated with the persistence of mononuclear phagocytes,
422 a family of cells that includes circulating monocytes, tissue-resident macrophages, and monocyte-
423 derived inflammatory macrophages (Nathan and Ding, 2010). VEGFR1 and PlGF are known to be
424 involved in the inflammatory pathways (Shibuya, 2015). Genetic ablation of the VEGFR1 TK domain in
425 mice allows normal vascular development but significantly suppresses VEGF-induced macrophage
426 migration (Hiratsuka et al., 1998). There is strong evidence that PlGF and VEGFR1 signaling can influence
427 how immune cells affect tumor growth and metastasis (Albonici et al., 2019; Ceci et al., 2020; Incio et
428 al., 2016; Kim et al., 2012; Muramatsu et al., 2010; Qian et al., 2015) as well as cardiovascular disorders
429 (Luttun et al., 2002; Raisky et al., 2007; Roncal et al., 2010) or rheumatoid arthritis (Murakami et al.,
430 2006).

431 PlGF/VEGF-A heterodimers have been detected in synovial fluid samples from patients with
432 inflammatory arthropathy and in human keratinocytes during wound healing, with levels of PlGF and
433 VEGF-A in synovial fluid correlating significantly with total leukocyte and neutrophil counts (Bottomley
434 et al., 2000). An increase in inflammatory cytokine production after VEGFR1 activation in mononuclear
435 phagocytes has also been observed in patients with rheumatoid arthritis, in which fibroblast-like
436 synoviocytes produce high levels of PlGF (Yoo et al., 2009). PlGF-induced VEGFR1 activation increased
437 TNF-α and IL-6 expression, whereas TNF-α and IL-1β upregulated VEGFR1 (Yoo et al., 2009).

438 In the retina, circulating monocytes, macrophages, and so-called resident microglia — which are
439 not true glial cells but specialized, resident mononuclear phagocytes —are constantly engaged in the
440 surveillance of their surrounding tissue (Akhtar-Schafer et al., 2018; McMenamin et al., 2019). Retinal
441 microglia are maintained mostly by self-renewal through the entire life span but can also be replenished
442 from extraretinal sources (Huang et al., 2018). This contrasts with continuous replenishment of
443 choroidal macrophages by circulating monocytes (O'Koren et al., 2019). During development, microglia
444 contribute to the refinement of the neural circuits (Akhtar-Schafer et al., 2018; McMenamin et al., 2019;
445 Reyes et al., 2017) and also influence morphogenetic patterning of the vascular network, facilitating
446 vascular anastomosis (Fantin et al., 2010; Kubota et al., 2009; Rymo et al., 2011). The VEGFR1 signal
447 contributes but is not indispensable for the development of retinal microglia and the superficial retinal
448 vascular networks (Ogura et al., 2017). In contrast, microglia in the deep retinal layer express VEGFR1,
449 and the decoy function of this receptor neutralized circulating PlGF/VEGF-A, thereby reducing
450 angiogenic branching of the deep retinal vessels (Stefater et al., 2011).

451 In addition to microglia, which are the most common immune cells in the retina, the retina also
452 contains ionized calcium-binding adapter molecule 1 (Iba1)-negative perivascular macrophages found on
453 the abluminal aspect of the vascular endothelial basal lamina that are closely associated with pericytes
454 and Müller cells in the deeper retina (Mendes-Jorge et al., 2009). The microglia and perivascular
455 macrophages can also be differentiated from one another since the macrophages express BM8 and
456 MOMA-2 antigen epitopes, which are not expressed by microglia (Mendes-Jorge et al., 2009) and their
457 position relative to retinal blood vessels indicate their involvement in the preservation of the BRB as
458 well as the immune defense against blood-borne pathogens (McMenamin et al., 2019). On breakdown
459 of the BRB and photoreceptor degeneration, the macrophages migrate to the site of damage as shown
460 in various models of retinopathy (Roubeix et al., 2019; Saban, 2018; Sennlaub et al., 2013). Retinal injury
461 can activate microglia and trigger the secretion of inflammatory mediators, such as CC chemokine ligand
462 2 (CCL2, also known as monocyte chemoattractant protein-1), IL-1β, IL-6, and TNF-α (Grossniklaus et al.,
463 2002; Oh et al., 1999), which can further aggravate retinal injury (Langmann, 2007).

464 In the disease state, activation of VEGFR1 results in the production by macrophages and
465 microglia of proinflammatory and proangiogenic mediators in the retina (Carmeliet et al., 2001; Crespo-
466 Garcia et al., 2017; Fischer et al., 2008; Rakic et al., 2003; Ziche et al., 1997a). Furthermore, PlGF may
467 stimulate VEGFR1-dependent migratory pathways of monocytes more efficiently than does VEGF-A
468 (Cicatiello et al., 2015; Clauss et al., 1996). Both activated microglia and monocyte-derived macrophages
469 are assumed to upregulate VEGFR1 in various sites including the retina (Barleon et al., 1996; Ogura et
470 al., 2017). VEGFR1 activation in these mononuclear phagocytes upregulates their production of pro-
471 inflammatory and pro-angiogenic cytokines, such as CCL2, IL-1β, IL-6, TNF-α, and VEGF-A (Murakami et
472 al., 2006; Selvaraj et al., 2003).

473 However, there remains a large gap in our understanding of how exactly VEGFR1 signaling in
474 inflammatory cells contributes to retinal vascular pathology. As outlined in this section, various types of
475 inflammatory cells of different origins (i.e., resident versus invading) and in different states of activation
476 are found in the retina. Thus, whilst certain features of the pathologies provide hints of inflammatory
477 cell involvement, further study of the many levels of intricacy might resolve some of the apparent
478 experimental paradoxes in the VEGFR1 literature.

479 **4. Role of VEGFR1 in retinal vascular disease**

480 The seminal discovery of increased VEGF levels in ocular fluids of patients with retinal eye diseases

481 (Adamis et al., 1994) introduced an era of anti-VEGF therapy in diseases such as DR, retinal vascular
482 occlusions, retinopathy of prematurity (ROP), neovascular AMD and others. Currently in use are, 1)
483 aflibercept and conbercept* (fusion proteins consisting of the ligand binding portions of VEGFR1 and
484 VEGFR2 extracellular domains fused to the Fc portion of human IgG), 2) bevacizumab* (a full-length
485 anti-VEGF monoclonal antibody), 3) ranibizumab (an anti-VEGF monoclonal antibody Fab fragment) and
486 4) brolucizumab (a single-chain antibody fragment), recently approved for neovascular AMD (Markham,
487 2019) (see **Table 1**).

488 Aflibercept and conbercept* bind all known VEGFR1 ligands, VEGF-A, PlGF, and VEGF-B, unlike
489 ranibizumab, bevacizumab,* and brolucizumab, which are VEGF-A specific (de Oliveira Dias et al., 2016;
490 Papadopoulos et al., 2012). It is tempting to hypothesize that some observed differences between the
491 clinical effects of anti-VEGF agents may result from differences in their targets, i.e. the binding of VEGF-
492 A, VEGF-B and PlGF versus just VEGF-A, but given the complexity of VEGFR1 signaling and the impact of
493 other factors like pharmacokinetics, binding affinities, and dosing strategies, this hypothesis remains
494 open to further investigation. Furthermore, despite significant advances in our understanding of the
495 molecular and cellular aspects of VEGF receptors and their ligands, it is only more recently that research
496 has focused on elucidating the effects of VEGFR1 versus VEGFR2 in the pathophysiology of retinal
497 diseases.

498 *4.1. Diabetic retinopathy*

499 Diabetic retinopathy is the leading vision-threatening disease in the working-age population
500 globally (Yau et al., 2012). Over years of hyperglycemic episodes, the accumulation of insults, including
501 advanced glycation end products and oxidative stress, damages retinal blood vessels and neural cells
502 (Duh et al., 2017). About one-third of diabetic patients display non-proliferative DR (NPDR)
503 characterized initially by intraretinal microvascular abnormalities and retinal microaneurysms, and
504 additionally by retinal hemorrhage, edema, and exudative lipoprotein deposits (known as hard
505 exudates) (Duh et al., 2017; Wong et al., 2018). In more severe cases, capillary non-perfusion and
506 subsequent tissue ischemia can lead to retinal microinfarctions and collateral vessel formation and
507 ultimately retinal neovascularization (Duh et al., 2017; Wong et al., 2018), thus evolving into PDR. PDR is
508 distinguished by the growth of retinal neovascularization extending into the vitreous cavity, ultimately

* Note on drug approval status: Bevacizumab is not approved for intraocular use, even though it is used off-label in several countries. Conbercept is currently only approved and used in China.

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509 resulting in vision-impairing vitreous hemorrhage and tractional retinal detachment (Duh et al., 2017;
510 Wong et al., 2018). A further complication is diabetic macular edema (DME), which affects central vision
511 in any DR, with an estimated prevalence of approximately 7% among people with diabetes (Yau et al.,
512 2012).

513 For DME treatment, intravitreally injected anti-VEGFs have been demonstrated to be effective in
514 multiple clinical trials and have subsequently been widely adopted worldwide (Ferrara and Adamis,
515 2016; Sivaprasad et al., 2017; Wong et al., 2018; Writing Committee for the Diabetic Retinopathy Clinical
516 Research et al., 2015). Anti-VEGF agents are effective in reducing the edema associated with the
517 increased vascular permeability in the retina and in improving the vision of patients with DME (Heier et
518 al., 2016; Nguyen et al., 2012). This clearly illustrates the pivotal role VEGF-A plays in angiogenesis as
519 well as in vascular permeability. However, the exact mechanisms behind this remarkable success story
520 are less well understood. Additionally, as outlined in the previous section, VEGF-A does not only play a
521 role in endothelial cell behavior but is also relevant for inflammatory cells, whose contribution to DR is
522 overshadowed by the current focus on vascular phenotypes. Furthermore, the roles of the different
523 VEGF ligands within the context of human retinal pathology have not yet been properly elucidated.
524 Nevertheless, there are clinical observations and animal experiments that allow us to hypothesize about
525 potential mechanisms.

526 Initiation of the inflammatory response that is linked to the early stages of the pathogenesis of
527 DR was demonstrated initially by leucocyte-mediated endothelial cell injury and death in animal models
528 (Joussen et al., 2001; Joussen et al., 2004). Indeed, one of the most compelling arguments for an
529 involvement of inflammation in DR is the well-established potency of corticosteroids in the treatment of
530 DME (Rittiphairoj et al., 2020; Whitcup et al., 2018; Wykoff, 2017). Interestingly, there is evidence that
531 benefits of steroid treatment are not limited to just edema but may also slow down development of PDR
532 and overall progression of DR (Pearson et al., 2011; Querques et al., 2017; Wykoff et al., 2017),
533 suggesting that inflammatory mechanisms (such as persistent low-grade inflammation) make a causal
534 contribution to DR (Kinuthia et al., 2020). This is consistent with a large collection of clinical studies
535 showing increased ocular levels of inflammatory mediators, including IL-1β, IL-6, IL-8, TNF-α, and CCL2,
536 in NPDR, DME and PDR (Bolinger and Antonetti, 2016; Chernykh et al., 2015; Funatsu et al., 2012; Kovacs
537 et al., 2015; Mao and Yan, 2014; Mesquida et al., 2019; Rubsam et al., 2018; Tang and Kern, 2011;
538 Wykoff, 2017; Zhou et al., 2012).

539 Considering the likely contributions from inflammatory cells in DR and the role of VEGF signaling

540 in inflammatory cells (as covered in section 3), a key emerging question is whether the benefits of anti-
541 VEGFs in ophthalmic practice are based only on the well-established effects on vessels or if they are also
542 acting on inflammatory cells, and beyond that, whether signaling via VEGFR1 may be relevant. For the
543 first part of this question there is considerable clinical evidence showing that anti-VEGFs can reduce
544 inflammatory cytokines in diabetic eye disease. For instance, aflibercept injections not only suppress
545 levels of VEGF-A in DME patients but also reduced inflammatory cytokines such as IL-6, IL-1β and others
546 (Mastropasqua et al., 2018). Similarly, a study on PDR patients receiving aflibercept showed reduced
547 levels of IL-6, IL-8, IL-10 and IL-1β in the vitreous (Raczyńska et al., 2018). Likewise, ranibizumab
548 treatment reduced levels of IL-1β, IL-8, IL-10, CCL2 and TNF-α in DME patients (Lim et al., 2018).
549 However, it is not known yet whether the changed cytokine levels are a direct result of VEGF signaling
550 inhibition or whether they are an indirect consequence of reduced vascular pathology.

551 The functional role of VEGFR1 in inflammation within the context of human diabetic eye disease
552 remains to be fully understood. Looking at the VEGFR1 specific ligand, PlGF, is certainly a path to
553 explore. In human eyes with DR, PlGF is elevated in addition to VEGF-A (Ando et al., 2014; Noma et al.,
554 2017; Noma et al., 2015), and there are significant increases in the levels of both VEGF-A and PlGF in
555 vitreous samples from eyes of patients with increasing levels of ischemia, i.e. from the normal to
556 diabetic state, or from PDR to neovascular glaucoma (Kovacs et al., 2015; Patel, 1989). Whilst the mere
557 presence of elevated PlGF levels in the vitreous does not prove a functional involvement in DR
558 pathology, there appears to be an association between PlGF levels and progressive disease severity in
559 DR and RVO (Noma et al., 2015). Mechanistic insights may be gained by comparing the clinical effects of
560 drugs that target specifically VEGF-A (e.g. ranibizumab) versus the ones that in addition also target the
561 VEGFR1 specific ligands PlGF and VEGF-B (e.g. aflibercept), although this is not straightforward either.
562 Current clinical trials usually focus on visual acuity or retinal thickness under therapy and therefore an
563 approach more focused on inflammation markers is required to shed more light on this topic.

564 An intriguing piece of clinical evidence comes, however, from a subtle feature in retinal OCT
565 images in patients with DME that might be useful for the assessment of inflammation in vivo. In some
566 patients with early DR, small well-demarcated, hyperreflective foci have been identified (Yu et al., 2019).
567 Such deposits are located within walls of intraretinal microaneurysms and in some cases distributed
568 throughout the retinal layers. Various etiologies have been suggested regarding the possible nature of
569 these hyperreflective foci and it is suggested that they represent lipoproteins or lipid-laden
570 macrophages, indicating extravasation and/or neuroinflammation, as an early subclinical sign of barrier

571 breakdown in DME (Bolz et al., 2009). In early stages of DME with few or no funduscopically visible
572 exudates, the number of hyperreflective spots, as visualized by OCT, decreased significantly after either
573 anti-VEGF or steroid treatment and correlated with functional data. DME with high number of
574 hyperreflective spots showed better morphologic and functional results (in terms of retinal sensitivity) if
575 treated, at least initially, with steroids versus a selective VEGF-A inhibitor (ranibizumab) (Frizziero et al.,
576 2016; Vujosevic et al., 2017). However, the hyperreflective spots cannot be used as a true proxy for
577 inflammation until their cellular nature has been established more firmly.

578 The target specificity of currently used anti-VEGF drugs might also yield some hints about the
579 pathobiological function of VEGFR1 in DR. There are numerous studies in DME patients showing
580 significant differences between ranibizumab and aflibercept when looking at certain clinical readouts,
581 with aflibercept showing higher efficacy or longer lasting treatment effects (Bhandari et al., 2020;
582 Jampol et al., 2016; Kaldirim et al., 2019; Ozkaya et al., 2020; Sarda et al., 2020; Shimizu et al., 2017).
583 Furthermore, comparisons between aflibercept and bevacizumab* had similar outcomes (American
584 Academy of Opthalmology, 2019; Virgili et al., 2018; Wells et al., 2015; Wells et al., 2016). For example,
585 some studies have found that aflibercept was statistically superior in vision gains compared to
586 ranibizumab and bevacizumab* (Protocol T) (Wells et al., 2015; Wells et al., 2016). These outcome
587 differences may be explained by several factors, such as anti-VEGF-A relative potency/binding affinity
588 (Papadopoulos et al., 2012), specificity for VEGF-A only versus VEGFR1 blockade through binding of
589 VEGF-A, PlGF and VEGF-B (Papadopoulos et al., 2012), duration of intraocular VEGF suppression (Fauser
590 and Muether, 2016; Fauser et al., 2014; Muether et al., 2013), ocular pharmacokinetics (Do et al., 2020;
591 Krohne et al., 2008; Krohne et al., 2012) and drug formulation. The higher binding affinity, multiple
592 molecular targets and ocular pharmacokinetics of aflibercept may be contributing factors to the
593 observed differences in clinical outcomes. While the respective relative importance of these factors is
594 unknown, these differences have been considered significant enough to define clinical practice
595 guidelines (American Academy of Opthalmology, 2019; Cheung et al., 2018b; Schmidt-Erfurth et al.,
596 2017). In this respect it will be interesting to see direct comparisons between aflibercept or conbercept*
597 and some of the more recently approved anti-VEGFs. For example, brolucizumab has a higher molarity
598 of VEGF-A binding sites than aflibercept but only binds VEGF-A and not PlGF and VEGF-B. Differences in
599 future clinical trial outcomes might inform us further about the molecular effects of these drugs.

600 Despite the current absence of such comparative data it is intriguing that aflibercept has been
601 shown to reduce overall DR progression in DME patients (Mitchell et al., 2018) and to improve DR

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602 severity in PDR patients (Nittala et al., 2020). This is remarkable because anti-VEGFs are generally seen
603 to treat complications of DR (i.e. high VEGF levels) and not the underlying disease. It is possible that
604 effects on inflammatory mechanisms could reduce overall progression and severity in DR. Future clinical
605 studies measuring cytokines in aqueous humor from DR patients treated with aflibercept or
606 conbercept* versus ranibizumab or brolucizumab are likely to add further insight here.

607 In addition to clinical observations, studies in animal models can inform us about potential
608 functional roles of VEGFR1 and PlGF in retinal pathology. For instance, overexpression of PlGF in ciliary
609 muscle of rats led to microaneurysms and vascular sprouts in the retinal vasculature, demonstrating the
610 pathogenic potential of elevated PlGF levels (Kowalczuk et al., 2011). Similarly, PlGF injection into the rat
611 eye vitreous caused sub-retinal fluid accumulation by opening RPE tight junctions (Miyamoto et al.,
612 2007). Additionally, deletion of the *Pgf* gene in a type 1 diabetic mouse model (*Ins2*Akita mouse carrying
613 a spontaneous point mutation in the *insulin 2* gene, (McLenachan et al., 2013)) led to protection from
614 capillary dropout, pericyte loss, and BRB breakdown (Huang et al., 2015). Furthermore, the absence of
615 PlGF increased Akt phosphorylation and inhibited the HIF1α–VEGF pathway, preventing retinal cell
616 death, capillary degeneration, pericyte loss, and BRB breakdown, which highlights the critical role of
617 PlGF and VEGFR1 in the development of DR. Increased expression of the tight junction molecule, ZO-1
618 and vascular endothelial-cadherin alongside sonic hedgehog and angiopoietin-1 also indicated additional
619 protection associated with *Pgf* deletion (Huang et al., 2015). In contrast, expression of intracellular
620 adhesion molecule (ICAM)-1, vascular cell adhesion molecule-1, CD11b, CD18 and retinal leukostasis
621 were not inhibited in this study (Huang et al., 2015).

622 An alternative approach, using VEGFR1 neutralizing antibodies in mice with streptozotocin-
623 induced diabetes, led to a reduction of leukostasis and various cytokines (including IL-1β), besides
624 lowered vascular permeability (He et al., 2015). This is consistent with another study in streptozotocin-
625 treated rats, demonstrating reduced TNF-α after intravitreally injected aflibercept (Lazzara et al., 2019).
626 Moreover, it has also been shown in type 1 diabetes mouse models that leukocyte and macrophage
627 infiltration was decreased by an anti-PlGF specific antibody or aflibercept, but not by VEGF-A specific
628 antibodies, suggesting a specific role of PlGF in retinal inflammatory mechanisms (Van Bergen et al.,
629 2017). One should keep in mind though, that models based on diabetic animals (typically rodents) have
630 limitations. Although they mimic some aspects of human NPDR, other features (such as PDR) are not
631 present. Furthermore, while these models can indicate what might be going wrong in human eyes, they
632 cannot be used to validate human disease mechanisms.

633 In histological analyses of human diabetic eyes, the loss of pericytes in the retinal vasculature is
634 one of the first cellular pathologies that has been recognized (Cogan et al., 1961), and it is assumed that
635 pericyte dropout is a key driver of vascular abnormalities in DR (Arboleda-Velasquez et al., 2015). In line
636 with this notion, pericyte loss can be seen in some animal models of diabetes (Robinson et al., 2012).
637 The consequences of pericyte loss in the retinal vasculature can also be studied in diabetes-independent
638 models. For example, pericyte recruitment to growing retinal vessels can be efficiently disrupted by
639 genetic or pharmacologic tools leading to disorganized vascular patterning with microaneurysms,
640 edema, and hemorrhage (Enge et al., 2002; Kitahara et al., 2018; Klinghoffer et al., 2001; Kusuhara et al.,
641 2018; Lindblom et al., 2003; Park et al., 2017; Uemura et al., 2002; Valdez et al., 2014) as shown in **Fig. 4,**
642 **A and B**.

643 Interestingly, in addition to the vascular pathologies, pericyte deficiency can also lead to
644 inflammatory phenotypes via the activation of nuclear factor of activated T (NFAT) cells in ECs, which
645 upregulates a series of inflammatory mediators and leukocyte adhesion molecules including CCL2 and
646 ICAM-1, resulting in influx of CCR2-expressing monocytes and perivascular infiltration of
647 CD45hiCD11b+Ly6C+ mononuclear phagocytes (**Fig. 4C**) (Ogura et al., 2017). These mononuclear
648 phagocytes, which might also comprise activated microglia, displayed amoeboid cell bodies with fewer
649 dendrites that physically contacted the denuded ECs (Ogura et al., 2017). In contrast to the tissue-
650 resident microglia, mononuclear phagocytes infiltrating into pericyte-deficient retinas exhibited
651 elevated VEGF-A, PlGF, and VEGFR1 (**Fig. 4, C-E**), which is indicative of VEGFR1 activation in an autocrine
652 or paracrine manner (Ogura et al., 2017). ECs devoid of pericytes revealed increased VEGFR2, which
653 would be expected to be activated by VEGF-A derived from mononuclear phagocytes and exacerbate
654 vascular hyperpermeability (Ogura et al., 2017). In *VEGFR1-TK-/-* mice, retinal edema and mononuclear
655 phagocyte infiltration were reduced even after pericyte depletion (**Fig. 4F**) (Ogura et al., 2017). In
656 addition, time-lapse imaging of ex vivo explants of pericyte-deficient retinas demonstrated that
657 aflibercept reduced the motility of mononuclear phagocytes and recovered their dendrite formation
658 (**Fig. 4G**) (Ogura et al., 2017). Furthermore, intravitreally injected aflibercept suppressed mononuclear
659 phagocyte infiltrations and vascular leakage in the pericyte-deficient retina (**Fig. 4H**). Together, as shown
660 in **Fig. 4I**, these observations indicate a positive feedback loop between ECs and mononuclear
661 phagocytes in pericyte-deficient retina, in which VEGFR1 signaling facilitates cell motility of
662 mononuclear phagocytes. Thus, despite the underlying complexity of multiple signaling pathways
663 mediating interactions between at least three different cell types, we can conclude that in this setting,

664 simultaneous neutralization of VEGF-A and PlGF can effectively block the cycle of BRB breakdown.

665 *4.2. Retinal vascular occlusions*

666 Retinal ischemia due to vascular occlusion occurs most commonly in branch retinal vein
667 occlusion (BRVO) and central retinal vein occlusion (CRVO) but may also appear as a complication in
668 hemoglobinopathies (sickle cell disease and thalassemia), peripheral ischemic retinopathies such as
669 Eales' disease, familial exudative vitreoretinopathy, sickle cell retinopathy, Susac syndrome and others
670 (Caprara and Grimm, 2012; Gilmour, 2015; Hartnett, 2017; Sigler et al., 2014). In all instances, key
671 features include elevated vascular permeability and edema, which can affect central vision (Ho et al.,
672 2016). Furthermore, sustained retinal ischemia can also result in the formation of new blood vessels
673 that grow toward the vitreous cavity without resolving retinal hypoxia (Fukushima et al., 2011; Ho et al.,
674 2016).

675 Clinically, ocular levels of VEGF-A and PlGF are elevated in RVO (Aiello et al., 1994; Noma et al.,
676 2015). An analysis of aqueous samples taken from controls and patients with BRVO showed marked
677 elevations of both VEGF-A and PlGF (Noma et al., 2014), and importantly a significant positive
678 correlation for both VEGF-A and PlGF with increasing levels of ischemia (Ryu et al., 2021). These positive
679 correlations are also observed in patients who have CRVO (Noma et al., 2015). Furthermore, significant
680 correlations were observed between levels of PlGF and soluble ICAM-1, PDGF-AA, CCL2, and IL-8 (Noma
681 et al., 2014, 2015), which implicates activation of microglia and macrophages by VEGFR1 as part of the
682 disease pathology in RVO patients.

683 As with DME, current treatment strategies for RVO include removal of the angiogenic and
684 inflammation drivers or the use of steroids to reduce the overall inflammatory response. Intravitreal use
685 of aflibercept and ranibizumab is effective in reducing edema and restoring visual acuity in patients with
686 BRVO (Campochiaro et al., 2015; Campochiaro et al., 2010) and CRVO (Boyer et al., 2012; Brown et al.,
687 2010; Holz et al., 2013). Furthermore, vision gains and maintenance of vision were reported in patients
688 with significant areas of non-perfusion as a result of CRVO in the COPERNICUS and GALILEO studies
689 (Feltgen et al., 2019; Pielen et al., 2017).

690 Clinical comparative studies show that agents that target VEGF-A only, or those that bind VEGF-
691 A and PlGF, both provide robust responses in improving vision and reducing macular edema. Clinical
692 study outcomes are particularly relevant, as any emerging signals are observed despite the inherent
693 variability between patients In a comparator trial in DME (Protocol T) (Wells et al., 2015; Wells et al.,

694 2016), statistical superiority in visual acuity gains was shown, and in RVO trials (LEAVO: (Hykin et al.,
695 2019); SCORE 2: (Scott et al., 2017)), less frequent treatment was required and fewer non-responders
696 were observed with aflibercept versus bevacizumab* and/or ranibizumab. In contrast, effects on
697 inflammatory readouts (e.g. aqueous CCL2, IL-6, IL-8 and others) appear to be similar (Cui et al., 2021;
698 Kotake et al., 2019). This is different from what we observed above in DR and might be due to the
699 different pathogenic contributions of PlGF signaling in RVO versus DR, or to differences in the
700 methodologies used to measure these agents in the aqueous.

701 The pathogenesis of RVO and its subtypes have been described in clinicopathological studies
702 (Green et al., 1981; Powner et al., 2016; Wolter, 1961), but its pathophysiology is less clear.
703 Nevertheless, attempts have been made to further explore potential pathobiological mechanisms of
704 RVO in animal models, typically using laser photocoagulation. Histological studies on primate RVO
705 models conducted in the 1970s have described the time course of degenerative changes of the vessels
706 and the surrounding tissue after occlusion (Hockley et al., 1976, 1979), which match human histology. In
707 addition, retinal vessel occlusion models have been generated and explored in many different animals,
708 usually with a focus on the vascular and edematous changes and retinal atrophy (Khayat et al., 2017).
709 Transcriptional profiling in rabbits and mice has shown a strong upregulation of genes associated with
710 hypoxia, angiogenesis, cell damage and inflammation (Martin et al., 2018; Neo et al., 2020) after
711 occlusion. In a non-human primate RVO model ranibizumab attenuated retinal edema and atrophy but
712 did not affect expression of CCL2, IL-6 and angiopoietin-1/2 (Inagaki et al., 2020), reflecting the clinical
713 findings mentioned above. On a cellular level, activation of microglia and invasion of macrophages from
714 the systemic circulation are prominent responses to experimental BRVO in mice (Ebneter et al., 2017).
715 Remarkably, the invading macrophages seem to have a protective effect on the vein ECs (VanderVeen
716 and Cataltepe, 2019), but whether VEGFR1 signaling is relevant upstream or downstream of the
717 inflammatory response, specifically in RVO, is not known.

718 *4.3. Retinopathy of prematurity*

719 ROP is a vasoproliferative disorder of premature infants born with an incompletely vascularized
720 retina. A mismatch between oxygen levels of the in utero and postnatal environments (exacerbated by
721 supplemental oxygen), can lead to delayed retinal vascularization, hypoxia and excessive angiogenic
722 stimuli in the peripheral retina. This results in abnormal vessel growth, in particular at the leading edge
723 of the developing retinal vasculature, and can cause loss of vision through macular dragging and

724 tractional retinal detachment (Mintz-Hittner et al., 2011). Clinically, ROP is classified according to the
725 extent of retinal vasculature development (zone I is the smallest, most central region, and zone III the
726 largest). Also relevant are the circumferential extent (described using hours of a clock face), the severity
727 (stage 1-5, with 5 being the most severe) and the presence of dilated, tortuous posterior pole vessels
728 (referred to as 'plus' disease) (Agarwal and Jalali, 2018; International Committee for the Classification of
729 Retinopathy of Prematurity, 2005).

730 The standard of care treatment for ROP is laser ablation of the peripheral - not yet vascularized -
731 retina, removing the source of the excessive angiogenic stimulus. Alternatively, anti-VEGFs can be used
732 to directly counteract the main angiogenic mediator in the retina, but this approach is still novel and its
733 place in the therapeutic armamentarium remains to be established (VanderVeen and Cataltepe, 2019) .
734 The BEAT-ROP study was the first large randomized trial and demonstrated a superiority of
735 bevacizumab* to laser treatment in recurrence rate and unfavorable outcome in zone I eyes, with the
736 caveat that this was not the case for zone II eyes and recurrence after laser treatment was unusually
737 high in this study (Mintz-Hittner et al., 2011; VanderVeen and Cataltepe, 2019). Nevertheless, in the
738 following years several smaller case series studies showed positive effects of bevacizumab* and
739 ranibizumab (Moran et al., 2014; Stahl et al., 2018; Stahl et al., 2019; Yang et al., 2018; Yoon et al.,
740 2017). Similarly, aflibercept has also been shown to be effective in ROP (Salman and Said, 2015), and
741 further clinical studies are underway assessing aflibercept versus laser treatment in ROP (e.g.
742 NCT04004208 [FIREFLEYE]). However, treatment decisions, such as laser versus anti-VEGFs or the
743 optimal timepoint for anti-VEGF injection, depend on multiple variables and are still not straight forward
744 (Stahl, 2018).

745 The effects of intravitreally injected anti-VEGFs can be clinically observed within a few days
746 when retinal vessels start to grow into the avascular peripheral retina. This apparently paradoxical, pro-
747 angiogenic effect of an anti-angiogenic drug can be explained by a normalization of excessively high
748 VEGF levels, which are known to prevent normal vascular development from studies in model systems
749 (Bentley et al., 2009; Gerhardt, 2008). However, correct dosage is clearly a crucial issue here as excess
750 inhibition of VEGF-A can prevent retinal vascularization as it has been shown in a canine model of ROP
751 (Lutty et al., 2011). To what degree VEGFR1 signaling might be relevant in ROP is however less obvious.
752 A retrospective study (single-center) compared ranibizumab versus aflibercept and found that the need
753 for retreatment was lower and recurrences were delayed in the aflibercept group (Sukgen and Kocluk,
754 2019). This hints towards an involvement of PlGF or VEGFR1 in ROP but more clinical studies are needed

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755 to draw firmer conclusions.

756 Nevertheless, some insights about the potential functions of VEGFR1 and PlGF in ROP have been
757 gained from animal models. The aberrant angiogenesis that develops in ischemic retinas can be
758 mimicked in neonatal mice or rats by exposing them temporarily to high atmospheric oxygen, which
759 leads to premature cessation of vascularization followed by an increase in retinal VEGF-A levels when
760 the animals are returned to room air and the development of oxygen-induced retinopathy(OIR) (Kim et
761 al., 2016; Smith et al., 1994). PlGF levels have also been shown to increase in OIR rodent models
762 (Ozgurtas et al., 2016; Sato et al., 2009). Notably, the formation of neovascular tufts is suppressed by
763 deletion of the *Pgf* gene or administration of an anti-VEGFR1 antibody in mouse OIR (Carmeliet et al.,
764 2001; Luttun et al., 2002). Furthermore, in comparison to a VEGFR2 specific antibody, the anti-VEGFR1
765 antibody was equally effective at reducing neovascularization and even more effective at preventing
766 BRB breakdown (Huang et al., 2011). It is unlikely that the effects of the anti-VEGFR1 antibody are based
767 on preventing VEGF-A from binding to VEGFR1 (i.e. inhibiting the endogenous VEGF-A inhibitor) and
768 raising VEGF-A levels, because it is established that rising VEGF-A levels in the OIR model worsen
769 neovascularization. It can therefore be assumed that the relevant mechanism here is the blocking of
770 direct VEGFR1 signaling, affecting either vascular leakage or inflammation. As discussed in sections 3.2
771 and 3.3, there is evidence for both scenarios.

772 It is well known that the neovascular response in the OIR model is accompanied by upregulation
773 of inflammatory signals, infiltration of mononuclear phagocytes and activation of resident microglia
774 (Binet et al., 2020; Brockmann et al., 2018; Davies et al., 2006; Sun et al., 2017; Wang et al., 2020). This
775 relates to preterm human infants where the risk of ROP is association with sepsis (Huang et al., 2019b)
776 and systemic inflammation (Lee and Dammann, 2012; Sood et al., 2010). More specifically, soluble
777 VEGFR1, IL-8, TNF-α and other inflammation-associated proteins in the serum were found to be
778 associated with increased risk in early ROP (Holm et al., 2017).

779 Experiments in the OIR model have shown that elimination of mononuclear phagocytes leads to
780 decreased neovascular tufts and facilitates vascular regeneration, demonstrating a functional role
781 (Kubota et al., 2009). The contribution of mononuclear phagocytes towards aberrant angiogenesis is
782 however unlikely to be mediated via VEGF-A, because macrophage-specific deletion of VEGF-A, HIF-1A,
783 or EPAS1 had no impact on VEGF-A levels in whole retinas, or on neovascularization in the OIR model
784 (Liyanage et al., 2016; Nürnberg et al., 2018). Nevertheless, mononuclear phagocytes might contribute
785 indirectly to VEGF-A upregulation via activation of Müller glia in ischemic retinas (Nürnberg et al., 2018).

786 In this setting, the specific functions of the VEGFR1 signaling in mononuclear phagocytes await
787 elucidation.

788 **5. Role of VEGFR1 in age-related macular degeneration**

789 The relationship between the eye and the immune system has often been considered one of
790 "immune privilege," in which a combination of the physical BRB and an inhibitory ocular
791 microenvironment (such as high levels of TGFβ) serve to limit local immune and inflammatory responses
792 in order to preserve vision (Zhou and Caspi, 2010). However, accumulating evidence suggests a role for
793 chronic inflammation in the pathogenesis of retinal diseases, including AMD and DR (Chen and Xu, 2015;
794 Guillonneau et al., 2017; Whitcup et al., 2013). So that we may further explore the role of VEGFR1 on
795 the migration and localization of inflammatory cells within the posterior eye with macular degeneration,
796 we should first fully understand those processes, schematically represented in **Fig. 1**.

797 In advanced disease conditions such as GA, in which there has been RPE death and the
798 photoreceptor cell layer shows signs of degeneration (Fleckenstein et al., 2018; Sarks, 1976), a
799 substantial body of evidence implicates the subretinal infiltration and accumulation of mononuclear
800 phagocytes (Combadiere et al., 2007; Eandi et al., 2016; Gupta et al., 2003; Hu et al., 2015; Lad et al.,
801 2015; Lavalette et al., 2011; Levy et al., 2015a; Levy et al., 2015b; Penfold et al., 2001; Sennlaub et al.,
802 2013). Mononuclear phagocytes also play a critical role in photoreceptor degeneration and the
803 recruitment and activation of inflammatory cells is thought to exacerbate photoreceptor cell death in
804 retinal degenerative conditions such as AMD (Akhtar-Schafer et al., 2018; Combadiere et al., 2007; Cruz-
805 Guilloty et al., 2013; Guillonneau et al., 2017; Kohno et al., 2013; Rutar et al., 2012; Sennlaub et al.,
806 2013; Suzuki et al., 2012). This is supported by findings from Bhutto et al. who showed that donor eyes
807 with AMD show increased numbers and degranulation of mast cells. It is speculated that mast cell
808 degranulation may, through release of proteolytic enzymes, contribute to death of the choriocapillaris
809 and the RPE and ultimately to CNV formation (Bhutto et al., 2016).

810 Deposits of soft drusen – lipoproteinaceous debris – within Bruch's membrane and adjacent to
811 the RPE are a known hallmark of early and intermediate AMD (Fleckenstein et al., 2018; Guillonneau et
812 al., 2017; Sarks, 1976), and represent a known risk factor for progression to advanced or late-stage AMD
813 (Klein et al., 2004). More recently, reticular drusen, observed as discrete yellow-white subretinal dots on
814 fundoscopy, have also been implicated in late AMD, as they appear to affect photoreceptor integrity and
815 are associated with RPE damage (Greferath et al., 2016). Interestingly, in late-stage AMD, presence of

816 reticular drusen is also associated with significantly thinner choroids (Cheung et al., 2018a; Thorell et al.,
817 2015), offering additional insights in the interplay of choroidal and retinal inflammatory processes as
818 AMD progresses. Both large classical drusen and reticular drusen are characterized by the accumulation
819 of mononuclear phagocytes in the subretinal space (Combadiere et al., 2007; Eandi et al., 2016;
820 Greferath et al., 2016; Guillonneau et al., 2017; Levy et al., 2015a; Sennlaub et al., 2013).

821 Mononuclear phagocytes have been identified in donor tissues using various markers: Ricinus
822 communis agglutinin-I (Gupta et al., 2003), C-X3-C motif chemokine receptor 1 (CX3CR1) (Combadiere et
823 al., 2007), CD18 (Combadiere et al., 2007; Levy et al., 2015a; Sennlaub et al., 2013), Iba1 (Sennlaub et al.,
824 2013), CD163 (Lad et al., 2015), and CD14 (Eandi et al., 2016). Although CX3CR1, CD18, and Iba1 are
825 expressed on ramified microglial cells, the presence of CD163-positive and CD14-positive mononuclear
826 phagocytes in AMD demonstrates an activation of microglial cells and/or the recruitment of monocyte-
827 derived macrophages, supporting the notion that AMD may be an inflammatory disease. Interestingly, a
828 significant portion of subretinal mononuclear phagocytes in GA, as well as in and around large drusen,
829 express CCR2, the receptor for the chemokine CCL2, which is only expressed on inflammatory
830 monocytes and early monocyte-derived macrophages (Sennlaub et al., 2013), indicating that the
831 subretinal infiltrate comprises a mixture of monocyte-derived macrophages and activated microglial
832 cells. McLeod et al. (2016) describe significantly increased numbers of Iba1-positive macrophages in the
833 choroid of eyes with signs of early and intermediate AMD. In addition, numbers of HLA-DR-positive
834 submacular macrophages were significantly increased in all stages of AMD, and they exhibited
835 morphologic features suggesting an activated state (McLeod et al., 2016). Debate remains regarding
836 how best to distinguish resident and infiltrating mononuclear phagocyte populations within the retina or
837 the choroid. A subset of mononuclear phagocytes may be clinically visible in AMD patients and a
838 significant number of mononuclear phagocytes (identified using immunohistological markers) in AMD
839 donor eyes contain melanosomes, presumably from ingested RPE debris (Lad et al., 2015; Sennlaub et
840 al., 2013).

841 The presence of hyperreflective foci is related to neovascular AMD severity (Altay et al., 2016). A
842 prospective, observational study conducted by the Age-Related Eye Disease Study 2 group found that
843 proliferation and inner retinal migration of hyperreflective foci detected by spectral-domain OCT were
844 correlated with the progression of AMD disease in terms of RPE atrophy and expansion of GA
845 (Christenbury et al., 2013; Leuschen et al., 2013). One could postulate that in addition to migrating RPE
846 cells, these cells could be melanin-containing mononuclear phagocytes (Zanzottera et al., 2015).

847 Hyperreflective foci are also reduced following anti-VEGF treatment; however, the heterogeneity of the
848 anti-VEGF treatment effects, as well as the mechanism behind this phenomenon, are currently unclear
849 (Coscas et al., 2013; Ota et al., 2010; Sennlaub et al., 2013). Thus, although it has been confirmed that
850 relevant inflammatory cells are implicated in the pathogenesis of AMD disease, these prior studies did
851 not explicitly examine whether VEGFR1 was expressed in these cells. Consequently, we must turn to
852 preclinical studies to assess the role VEGFR1 may play.

853 Despite major differences between the murine model and human disease processes, laser-
854 induced CNV in the mouse model is used extensively in retinal research as it does mimic the main
855 phenotypical features of exudative AMD (Akhtar-Schafer et al., 2018; Tsutsumi et al., 2003). The laser
856 ruptures the RPE layer and Bruch's membrane causing a rapid recruitment of mononuclear phagocytes,
857 and, within a few days, choroidal capillaries penetrate into the retina (Akhtar-Schafer et al., 2018). The
858 laser CNV model has been used to demonstrate that blocking inflammatory macrophage recruitment
859 strongly reduces CNV (Akhtar-Schafer et al., 2018; Tsutsumi et al., 2003). The role of VEGFR1 itself was
860 recently shown in the development of CNV post-laser treatment using a tetrameric tripeptide, called
861 iVR1, directed against the receptor and specifically developed as an antagonist of VEGFR1 (Tarallo et al.,
862 2020). Intravitreal administration of iVRI potently inhibited laser-induced CNV in a dose-dependent
863 manner seven days after laser-included damage (reduction of CNV volume by around 70%, p=0.0002
864 compared to DMSO control injections). Remarkably, this treatment was more effective than an anti-
865 mouse VEGF-A polyclonal antibody (which achieved a reduction of CNV volumes of around 50%,
866 p=0.001). The observation points to the involvement of VEGFR1 in pathological neovascularization and
867 therefore blocking this receptor provides a potential alternate treatment route besides VEGF blockade.
868 Interestingly, when a chemically slightly modified version of iVRI was administered by gavage, a
869 significant reduction in CNV was also observed (around 50% of CNV volume, p=0.001).

870 Macrophage-derived VEGF-A does not contribute significantly to CNV development in mice
871 (Huang et al., 2013; Liyanage et al., 2016). However, VEGF-A can promote mononuclear phagocyte
872 recruitment and infiltration in models of laser-induced CNV (Balser et al., 2019). This effect could be
873 due, in part, to the stabilizing effect of VEGF inhibition on the local vasculature as well as to a direct
874 effect of VEGF-A on mononuclear phagocytes. Indeed, circulating human monocytes, which participate
875 in mononuclear phagocyte infiltration in AMD, express VEGFR1 but not VEGFR2, and a VEGFR1-
876 neutralizing monoclonal antibody has been shown to significantly suppress VEGF-A-induced migration of
877 microglial cells (Sawano et al., 2001). In addition, Massena et al. described a specific population of

878 neutrophils, characterized as CD49d+/CXCR4-high/VEGFR1-high, that migrate to hypoxic areas,
879 potentially enhancing angiogenesis (Massena et al., 2015). This process is dependent on neutrophil
880 VEGFR1 and endothelial VEGFR2 expression. Recruited neutrophils co-express CD49d, CXCR4, and
881 VEGFR1 and use VLA-4 integrin to facilitate extravasation: VEGFR2 is not expressed by this specific, pro-
882 angiogenic population (Massena et al., 2015).

883 Similarly, a comparison of an RNA sequencing database (Immunological Genome Project,
884 immgen.org) revealed that monocytes, but not microglial cells, express significant amounts of VEGFR1,
885 and neither cell expresses VEGFR2, in this analysis, at least in mice. Consistent with the role of VEGFR1
886 in monocyte recruitment to the laser-injured subretinal space is the observation that the blockade of
887 PlGF (Crespo-Garcia et al., 2017) and VEGFR1, but not VEGFR2, inhibits the peak of mononuclear
888 phagocyte recruitment three to four days following laser injury (Huang et al., 2013). The inhibitory
889 effect of VEGFR2-blockade at 14 days after laser impact (Huang et al., 2013) is likely due to the direct
890 effect of VEGF-A/VEGFR2 on vascular permeability.

891 Two recent studies investigated the role of PlGF and VEGF-A inhibition on neovessel formation
892 and mononuclear phagocyte reactivity in the murine laser-CNV model (Balser et al., 2019; Crespo-Garcia
893 et al., 2017). Both reports showed that PlGF inhibition, particularly with aflibercept, dampened vascular
894 leakage and CNV. Importantly, blocking PlGF and VEGF-A, but not VEGF-A alone, prevented the
895 accumulation of reactive microglia and macrophages in the lesion area. Higher levels of PlGF and VEGF-A
896 were detected in the laser-damaged retina by immunostaining, and in situ co-expression of PlGF and
897 VEGF-A was demonstrated by the presence of Iba1-positive mononuclear phagocytes in the RPE/choroid
898 complex. These data were verified by quantitative enzyme-linked immunosorbent assays, again
899 demonstrating a strong induction of VEGF-A and PlGF protein levels in the laser-CNV model and
900 effective inhibition of both factors, especially with aflibercept (**Fig. 5**) (Balser et al., 2019).

901 Use of intravitreally administered anti-VEGF agents has been associated with a reduction in
902 intraocular (aqueous humor/vitreous humor) levels of selected pro-inflammatory cytokines that are
903 produced by mononuclear phagocytes (Noma et al., 2017), which could indicate that inhibiting VEGF
904 reduces the inflammation in human patients. AMD patients treated with intravitreal ranibizumab or
905 aflibercept displayed a reduction in aqueous humor levels of inflammatory factors and VEGF-A, which
906 accompanied improvements in visual acuity and central macular thickness (Motohashi et al., 2017).
907 Furthermore, some studies have shown increased growth factors and pro-inflammatory mediators after
908 anti-VEGF treatment in subpopulations of patients categorized as low- to non-responders

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909 (Pongsachareonnont et al., 2018), suggesting that there is complexity in the underlying mechanisms of
910 inflammation, and in the identification and role of relevant mononuclear phagocytes.

911 In summary, these reports suggest that the main effect of VEGF-A and PlGF on subretinal
912 inflammation is the participation of VEGFR1 signaling in monocyte recruitment and activation of retinal
913 mononuclear phagocytes. In human AMD, indirect evidence suggests that a similar VEGF-A/PlGF-
914 mediated mechanism might contribute to the pathogenic retinal inflammation. Aqueous humor
915 concentrations of CCL2, which is expressed by subretinal mononuclear phagocytes in AMD (Grossniklaus
916 et al., 2002; Sennlaub et al., 2013) and implicated in inflammatory monocyte recruitment, is reduced
917 following two months of anti-VEGF therapy (Motohashi et al., 2017). In addition, anti-VEGF-A/anti-PlGF
918 therapy reduces the number of hyperreflective foci that likely, represent pigment-laden infiltrating
919 mononuclear phagocytes, at last in part (Coscas et al., 2013). Taken together, the inhibition of VEGFR1
920 signaling might contribute to the beneficial effects of anti-VEGF-A/anti-PlGF in AMD, as it is likely to help
921 control the pathogenic inflammation.

922 Results of a number of randomized clinical studies comparing anti-VEGF-A therapies with the
923 anti-VEGF-A/anti-PlGF therapy have been published, but it remains unclear whether there are distinct
924 differences between therapies in terms of vision improvements or anatomical benefits. One recent
925 meta-analysis of observational studies with wet AMD patients suggests that an anti-VEGF-A/anti-PlGF
926 strategy may be more beneficial for those patients with initial reduced vision (Zhang et al., 2017), which
927 would likely be a more difficult-to-treat population. This would suggest a potential benefit of VEGFR1
928 inhibition by agents that block all VEGFR1 ligands including PlGF, such as aflibercept and conbercept*,
929 versus other anti-VEGFs such as ranibizumab, bevacizumab* or brolucizumab, which do not bind PlGF. It
930 has been established that inflammation and hypoxia are important contributors to retinal vascular and
931 choroidal diseases. Notably, inflammatory cells such as microglia and monocytes upregulate VEGFR1,
932 and it has also been shown that VEGFR1 is the sole VEGF-A receptor upregulated during hypoxic
933 conditions (Miyamoto et al., 2007). There is a clear need in this area to better understand, from the
934 clinical perspective, the interplay between microinflammation and angiogenesis in CNV. Furthermore,
935 studies that examine the interplay of target binding affinity and potency, intraocular half-life and
936 duration of VEGF suppression within the eye may provide further context to assign relative order of
937 importance for understanding clinical differences in efficacy among the agents.

938 6. Future directions

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939 To date, the use of VEGF inhibitors has revolutionized the treatment of degenerative retinal
940 diseases characterized by neovascularization, including AMD and DR, with many of these agents coming
941 to represent new standards of care (Bakri et al., 2019; Framme et al., 2018; Singh et al., 2019; Tsilimbaris
942 et al., 2016). Estimates suggest that more than 90% of patients with AMD treated with anti-VEGF
943 therapy avoid moderate to severe vision loss (Miller, 2016). However, despite these therapeutic
944 successes, a subset of patients fails to respond to anti-VEGF therapy or show sub-optimal or a
945 diminishing response over time (Nguyen et al., 2018). It is therefore important to understand the
946 mechanistic basis of these shortcomings so we can continue to raise therapeutic standards. As our
947 understanding of the pathogenesis of ocular neovascular diseases continues to evolve and potential new
948 therapeutic targets and/or formats of anti-VEGF mediated mechanisms of action are being identified,
949 the hope is that this progress can be translated into the clinical setting (**Table 1**).

950 *6.1. Receptor–ligand interrelationships and localization*

951 One of the biggest challenges in this field remains the lack of clarity around the functional
952 relevance of ligand and receptor heterodimers versus homodimers. As discussed in this review, it is
953 possible for heterodimers to have stimulatory as well as inhibitory effects depending on the
954 circumstances. Unless we fully understand the full range of relevant molecular interactions between
955 VEGF-A, VEGF-B and PlGF, we will continue to struggle with the interpretation of experimental
956 manipulations and clinical observations. Furthermore, it is essential to gain a clearer picture about which
957 cells send signals and which cells receive them, including within inflammatory cell subpopulations, and
958 further experimental research is clearly needed here. In this context it is important to keep in mind that
959 there may be important differences between animal models and humans with regards to the prevalence
960 of isoforms and heterodimers in the VEGF ligand/receptor family as well as immune cell subpopulations.
961 It is likely that the rapidly evolving technologies enabling integrative single-cell analysis (Stuart and
962 Satija, 2019) will be transformational in this field.

963 *6.2. Research models*

964 We have discussed the complexity of the pathogenic processes in this review and identified
965 some of the multifactorial interactions between different cell types. An ongoing need exists for new, or
966 modified, research models with which to explore those cellular interactions and their impact on
967 neovascular disease and retinal and choroidal vascular permeability disorders. Although existing

968 preclinical models have proven helpful – and new models continue to emerge (Kitahara et al., 2018;
969 Morita et al., 2018) – they never fully represent a given human pathology. An overreliance on animal
970 models that phenocopy particular traits of human eye diseases (e.g. neovascularization) may arguably
971 be at least in part responsible for the high failure rate at the transition from preclinical proof of concept
972 to clinical application. Instead, models that aim to improve our understanding of specific molecular
973 interactions and signaling pathways, which have been validated as relevant in humans, are more likely
974 to advance therapeutic development.

975 *6.3. Inflammation*

976 We have also seen that questions remain regarding VEGFR1 in the context of inflammatory cells
977 in ocular disease processes. These concern the potential differentiating effects of blocking PlGF versus
978 VEGF-A or VEGF-B on inflammatory processes; the specific role of VEGFR1 on microglia/macrophages
979 (Ding et al., 2018) in terms of downstream signaling; the possible role of co-receptors; the composition
980 and dynamics of different immune cell subpopulations; and the consequences of selective targeting with
981 pharmacological agents. Recent human data have implicated inflammatory processes in AMD and DR,
982 with high intraocular levels of VEGF family ligands (e.g. VEGF-A and PlGF) and pro-inflammatory
983 cytokines (e.g. TNF-α and CCL2) reported (Ten Berge et al., 2019; Tsai et al., 2018), although the impact
984 of immunomodulation at the cellular level remains unclear. In patients with various retinal diseases,
985 only a limited number of studies have examined the effects of anti-VEGF treatment on aqueous/vitreous
986 levels of selected pro-inflammatory cytokines (Motohashi et al., 2017; Noma et al., 2017). Further
987 robust studies are required to clarify the complex relationships between intravitreal VEGF-A or PlGF
988 inhibition and effects on downstream mediators quantified in aqueous/vitreous media.

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989 **7. Conclusions**

990 This review has delved into the context and role of VEGFR1 in retinal and choroidal vascular
991 diseases, providing updates on the requisite preclinical studies and clinical context that inform on
992 emerging evidence of potentially greater contributions by VEGFR1 to these mechanisms than were
993 previously recognized. Signal transduction following VEGF-A, VEGF-B, or PlGF binding to relevant VEGFRs
994 was examined in the context of known, versus yet to be elucidated, downstream activity. Preclinical
995 studies have shown that VEGFR1 acts as a decoy receptor, and that transduction signaling following
996 VEGF-A versus PlGF binding to VEGFR1 may demonstrate attenuated versus potentiated signaling. Thus,
997 further work is merited to clarify the differences and the downstream consequences of these
998 differences in retinal diseases.

999 Advances have been made in the understanding and the importance of VEGF-A/PlGF
1000 heterodimers, VEGFR1/VEGFR2 heterodimers versus homodimeric ligands and/or homodimeric VEGF
1001 receptors in the context of the pathobiology of retinal diseases and other relevant organ systems. It
1002 remains challenging, however, to show clear clinical benefit to specific ligand/receptor interactions in
1003 retinal disease function. Greater understanding will require more nuanced studies to interpret and
1004 establish robust relationships.

1005 Both preclinical and clinical studies in retinal vascular diseases such as diabetic eye diseases,
1006 RVO and ROP have provided support and understanding for the contributory role of inflammation
1007 mediated by VEGFR1/PlGF/VEGF-A. The results of well-designed comparative trials may provide insights
1008 regarding the functional benefit of specific anti-VEGF agents with different target profiles. As discussed
1009 in section 4, in retinal vascular diseases such as DME and RVO some trials have shown outcome
1010 differences between aflibercept and other agents that bind VEGFA only, which has led to speculation
1011 concerning a potential role for PlGF inhibition contributing to these differences. Further characterization
1012 of the molecular features and pharmacokinetic profiles for these agents may contribute to our
1013 understanding and support our quest to explain these clinical differences. In choroidal vascular diseases,
1014 preclinical evidence is just beginning to clarify a role for VEGFR1/PlGF in their pathobiology (Balser et al.,
1015 2019; Crespo-Garcia et al., 2017). Prior mechanistic work in multiple studies has confirmed that
1016 mononuclear phagocytes contribute to the pro-inflammatory environment within AMD (Kauppinen et
1017 al., 2016; Knickelbein et al., 2015). It remains to be determined whether VEGFR1 was induced in these
1018 mononuclear phagocytes which, upon binding to VEGF-A or PlGF, in turn contributed to the
1019 inflammatory milieu described by such studies. Even as experiments have confirmed the major

1020 contribution of inflammation to the pathology of AMD, most studies have not examined whether
1021 VEGFR1 was induced in the mononuclear phagocytes. Thus, an opportunity remains to better
1022 understand the potential interplay between known complement cascades within the retina and VEGFR1-
1023 induced inflammatory cytokines, and to further examine and/or establish a role for VEGFR1/PlGF and its
1024 clinical relevance in AMD.

1025 **Funding**

1026 Medical writing, illustration, and editorial assistance (under the direction of the authors) was
1027 provided by Apothecom, UK, and funded by Bayer Consumer Care AG (Switzerland).

1028

1029 ***** Note on drug approval status: Bevacizumab is not approved for intraocular use, even though it is used
1030 off-label in several countries; Conbercept is currently only approved and used in China.

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1032 **Table 1.** Currently approved and emerging anti-VEGF therapies and ongoing clinical trials**.**
1033

Drug	Molecular Features	Approval Status	Ongoing Trials
Ranibizumab (LUCENTIS)	Fab against all VEGF-A isotypes	First approval 2004 Multiple retinal disease indications	LUMINOUS – observational study Control arms for multiple emerging anti-VEGFs and other targets
Aflibercept (EYLEA)	Fusion protein against all VEGF-A isotypes, VEGF-B and PlGF	First approval 2012 Multiple retinal disease indications	Multiple observational studies Control arms for multiple emerging anti-VEGFs and other targets
Brolucizumab (BEOVU)	scFv against all VEGF-A isotypes	First approval 2019 nAMD USA	Multiple trials initiated/planned in DME, RVO, and T&E trials in nAMD
Conbercept	Fusion protein against all VEGF-A isotypes and PlGF	Approval (China) 2013 nAMD	Phase III
Abicipar	DARPin against all VEGF-A isotypes	Phase III	Phase III development in nAMD (not approved by FDA [June 2020])
Faricimab	Antibody against VEGF-A and anti-Ang2	Phase III	Phase III development in nAMD and DME

1034 Ang2, angiopoietin-2; DARPins, designed ankyrin repeat proteins; DME, diabetic macular edema;
1035 Fab, monoclonal antibody fragment; nAMD, neovascular age-related macular degeneration; PlGF,
1036 placental growth factor; RVO, retinal vein occlusion; scFv, single-chain variable fragment; T&E, treat and
1037 extend; VEGF-A, vascular endothelial growth factor-A.
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1039 **Figure legends**

1040 Figures 1-4 are to be reproduced in color

1041 **Fig. 1.** Schematic representation of VEGFR1 in the choroid and retina and VEGFR1 signaling (for
1042 illustrative purposes and not to scale). **A** shows VEGFR1 expression in various types of cells, including
1043 vascular endothelial cells, pericytes, mononuclear phagocytes, Müller cells, photoreceptor cells, and the
1044 retinal pigment epithelium. **B** shows VEGFR1 signaling through VEGF-A and/or PlGF, via a variety of
1045 different pathways, contributing to numerous pathologic processes in the choroid and retina. In
1046 endothelial cells and pericytes: pericyte ablation, loss of tight junctions between endothelial cells,
1047 vasodilation, breakdown of the blood-retinal-barrier, increased permeability and leakage, edema and
1048 hemorrhage in surrounding tissue, neutrophil migration and monocyte migration and differentiation
1049 into macrophages, influx of pro-inflammatory cytokines e.g. tumor necrosis factor-α and interleukin-6,
1050 into surrounding tissue, increased angiogenic sprouts and neoangiogenesis. **C** shows consequences of
1051 excess VEGFR1 signaling in the choroid and retina: in retinal pigment cells: neoangiogenesis of vessels
1052 through Bruch's membrane into retinal pigment epithelium, loss of retinal pigment cells; in
1053 photoreceptor cells: loss of photoreceptor integrity, rod death and cone segment loss; in Müller cells:
1054 Müller cell activation; in microglial cells: recruitment, accumulation, and activation of microglial cells
1055 and other retinal macrophages, release of pro-inflammatory cytokines e.g. platelet-derived growth
1056 factor-A, soluble intracellular adhesion molecule-1, CC chemokine ligand 2, and interleukin-8, leading to
1057 the development of hyperreflective foci.

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1059 **Fig. 2.** VEGFR1 and VEGFR2 and the family of ligands and co-receptors. There are five VEGFR
1060 ligands, of which VEGF-A binds to both VEGFR1 and VEGFR2 and PlGF only binds VEGFR1. Splicing
1061 creates isoforms of both VEGF-A and PlGF. In addition, soluble/secreted versions of VEGFR1 and VEGFR2
1062 can be produced via alternative splicing or proteolytic cleavage retaining the extracellular ligand–binding
1063 domains. Furthermore, VEGF-A and PlGF are able to bind neuropilin (NRP) 1 and 2, bridging VEGFRs and
1064 NRP1 or NRP2 to create holoreceptor complexes. VEGF-A and PlGF ligands and the VEGFR1 and VEGFR2
1065 receptors can form heterodimers as well as homodimers. Functional synergistic effects of PlGF and
1066 VEGF-A are due to sharing of the common receptor, VEGFR1, and the ability to heterodimerize.

1067

1068 **Fig. 3.** Pgf-DE-Ki mice, a fully functional Pgf-KO mouse achieved by knocking in the Pgf-DE variant

1069 unable to bind and activate VEGFR1, show robust reduction of CNV and protection from vascular
1070 leakage. **A** is CNV volumes measured 7 days after laser-induced damage by Isolectin B4 staining of RPE-
1071 choroid flat mounts. **B** is qualitative fundus fluorescein angiography in C57BL6/J, Pgf-KO, and PlGF-DE-
1072 Knock-in mice acquired at three different times (early – 1 min, intermediate – 5 min, late – 15 min) after
1073 intraperitoneal delivery of fluorescein at days 3, 7, and 14 after laser-induced damage.
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1075

1076 **Fig. 4.** VEGFR1 signal in a mouse model of pericyte-deficient retinopathy (pups intraperitoneally
1077 injected with an anti-platelet-derived growth factor receptor β monoclonal antibody [clone APB5 in **A–**
1078 **H**] or control phosphate-buffered saline **[A–E]**) at postnatal day [P]1). **A.** Labeling of retinal endothelial
1079 cells (ECs) and pericytes (PCs) at P5 by whole-mount immunohistochemistry (WIHC) for CD31 and NG2,
1080 respectively. Note the absence of PCs and disorganized vascular networks in the APB5-treated retina. **B.**
1081 Hematoxylin and eosin (HE) staining of paraffin sections from P10 retinas showing edema and
1082 hemorrhage in the APB5-treated retina. **C.** Flow cytometry in P8 retinas. Tissue-resident microglia and
1083 inflammatory mononuclear phagocytes (MPs) are represented by $CD45^{lo}CD11b^{+}$ and $CD45^{hi}CD11b^{+}$ cells,
1084 respectively. Note the high VEGFR1 expression level in $CD45^{hi}CD11b^{+}Ly6C^{+}$ MPs from the APB5-treated
1085 retinas. **D.** Retinal whole-mount in situ hybridization for *Vegfa* (left) and *Pgf* (right) at P8 in conjunction
1086 with labeling of vascular basement membranes and MPs by WIHC for type IV collagen and Iba1,
1087 respectively. Note the upregulation of *Vegfa* and *Pgf* in perivascular MPs of the APB5-treated retinas. **E.**
1088 VEGFR1 reporter expression in P8 retinas from *Vegfr1-BAC-DsRed* mice in conjunction with WIHC for
1089 Iba1. Note the VEGFR1-expressing MP (arrowhead) in the APB5-treated retina. **F.** Retinal cups (upper)
1090 and WIHC for CD31 and Iba1 (lower) at P11 in APB5-treated *VEGFR1-TK* mice. Note the suppression of
1091 retinal edema and MP infiltration even without PC coverage in *VEGFR1-TK*$^{-/-}$ mice. The graphs show the
1092 number of $Iba1^{+}$ cells per area and the vessel density (n = 20). **G.** The trajectory of MPs in APB5-treated
1093 retinas from P8 *Cx3cr1-GFP* mice. After 3 hours ex vivo imaging, retinas were treated with control IgG or
1094 aflibercept, and further monitored for 3 hours. The graphs show quantification of cell body movement
1095 velocity (Pre IgG, n = 68; Post IgG, n = 56; Pre VEGF Trap, n = 52; Post VEGF Trap, n = 47) and total
1096 dendrite length per cell (Pre IgG, n = 40; Post IgG, n = 34; Pre VEGF Trap, n = 43; Post VEGF Trap, n = 33).
1097 **H.** Labeling for isolectin B4 (IB4), ICAM-1, and Iba1 at P13 in APB5-treated retinas after intravitreal
1098 injections of control IgG or aflibercept at P7. Note the normalization of vascular networks with reduced
1099 MP infiltration after aflibercept injection. **I.** Schematic diagram of EC-MP interactions in PC-deficient

1100 retina. In ECs, activation of nuclear factor of activated T cells (NFAT) leads to upregulation of CCL2,
1101 which subsequently facilitates the influx of circulating $CCR2^+$ monocytes. The infiltrating monocytes and
1102 activated microglia contribute to generation of inflammatory MPs, which secrete VEGF-A and PlGF, and
1103 activate VEGFR1 in MPs and VEGFR2 in ECs. The VEGF-A-VEGFR2 signal further activates NFAT. This
1104 positive feedback loop sustains breakdown of the blood-retina barrier. In box-and-whisker plots, median
1105 (line within the box), upper and lower quartile (bounds of the box), with minimum and maximum values
1106 (bars) are shown. $^{***}p<0.001$; NS, not significant, by Student's *t*-test. Scale bars, 50 μm (A and B); 20 μm
1107 (D and E); 100 μm (F and H). Adapted from Ogura et al., 2017 with permission from American Society for
1108 Clinical Investigation.

1109

1110 **Fig. 5.** Effects of PlGF and VEGF inhibition on mononuclear phagocytes in retinal flat mounts in
1111 the laser-induced mouse model of CNV. **A and B** are quantification of microglia/macrophages per laser
1112 spot in retinal flat mounts 3 and 7 days, respectively, after laser-induced damage. **C and D** are
1113 quantification of ionized calcium-binding adaptor molecule 1 signals 3 and 7 days, respectively, after
1114 laser coagulation in retinal flat mounts by counting the mean of colored pixels per image. **E, F, and G** are
1115 interleukin-6, interleukin-1β, and tumor necrosis factor levels, respectively, in retinal flat mounts 6 hours
1116 after laser damage quantified by enzyme-linked immunosorbent assay with naive (not lasered) animals
1117 used as controls. Reproduced under Creative Common CC-BY license (Balser et al., 2019).

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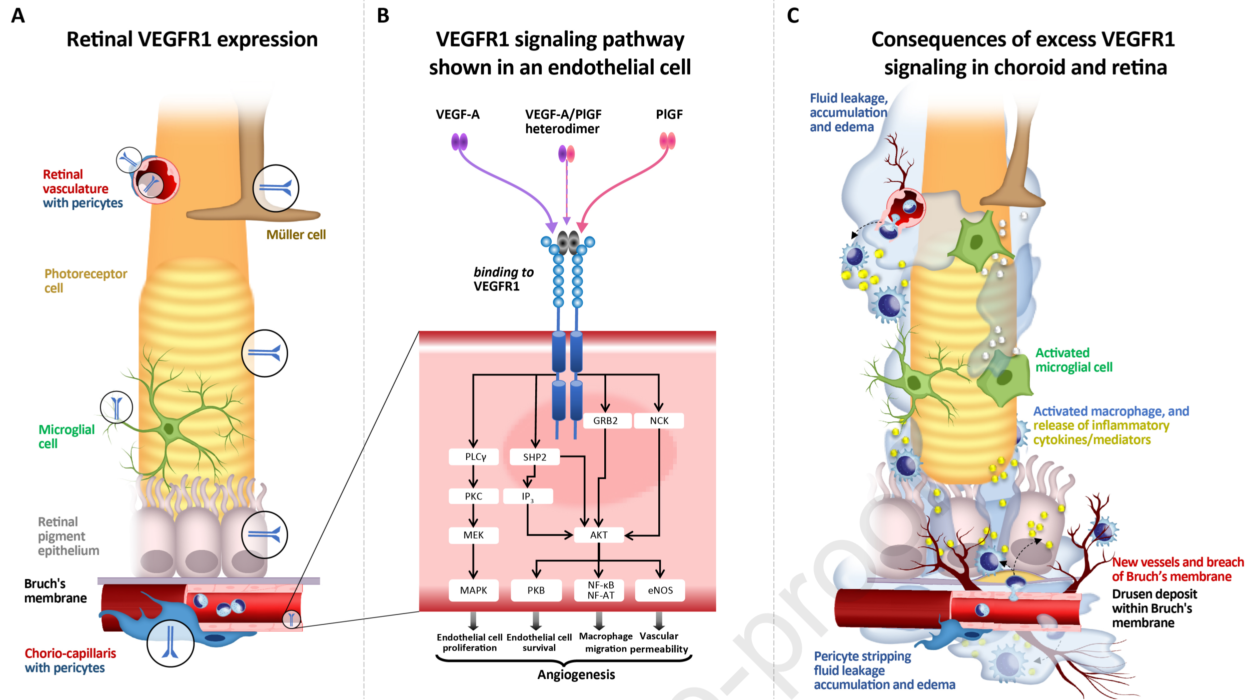

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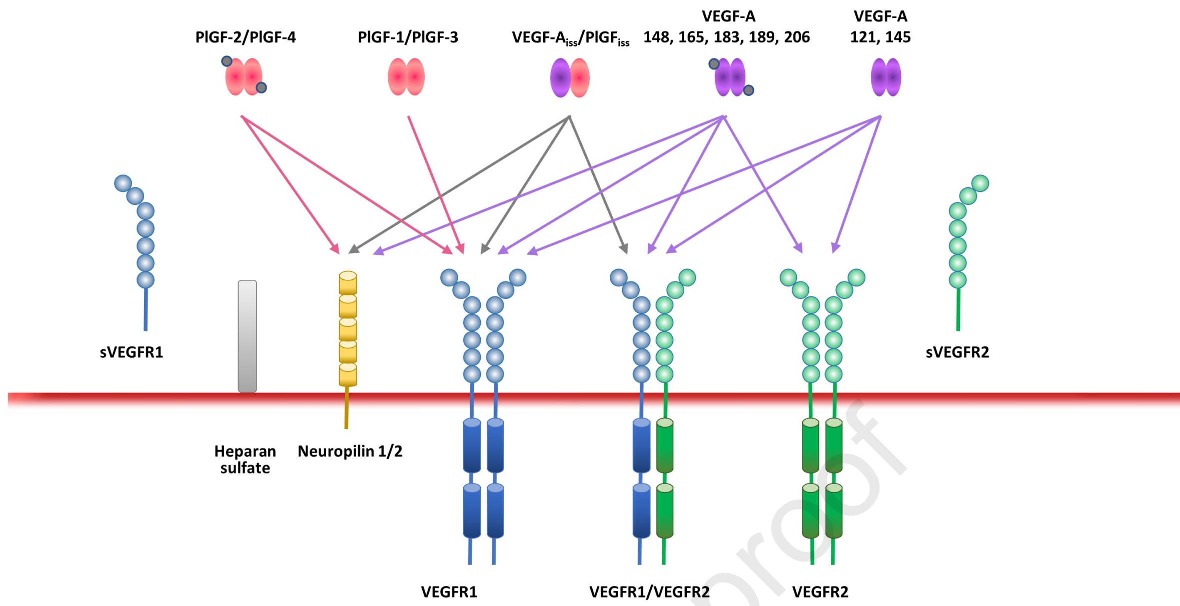

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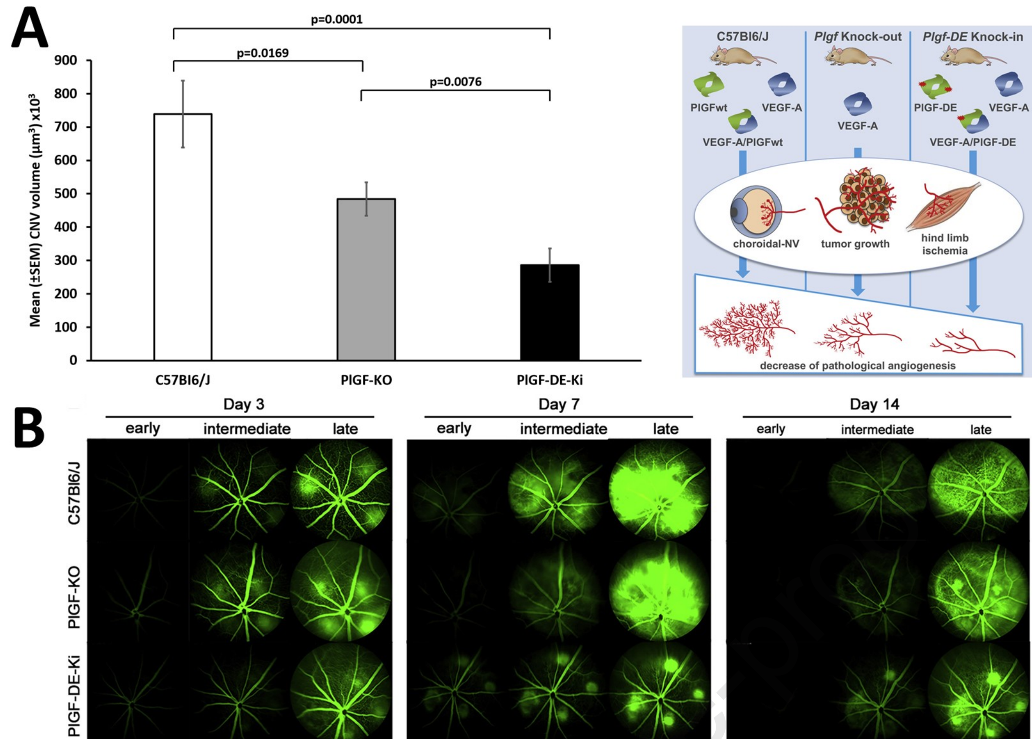

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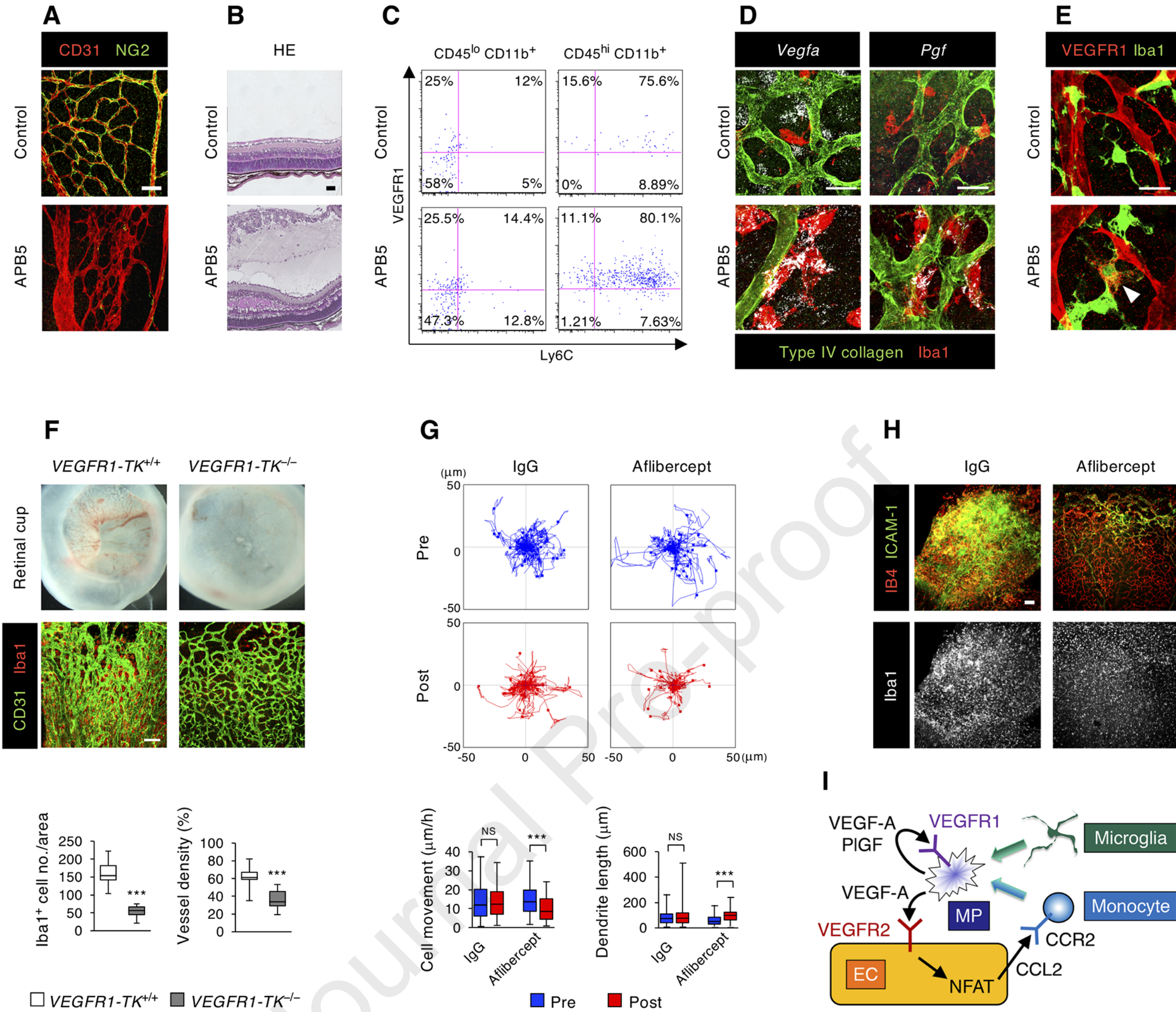

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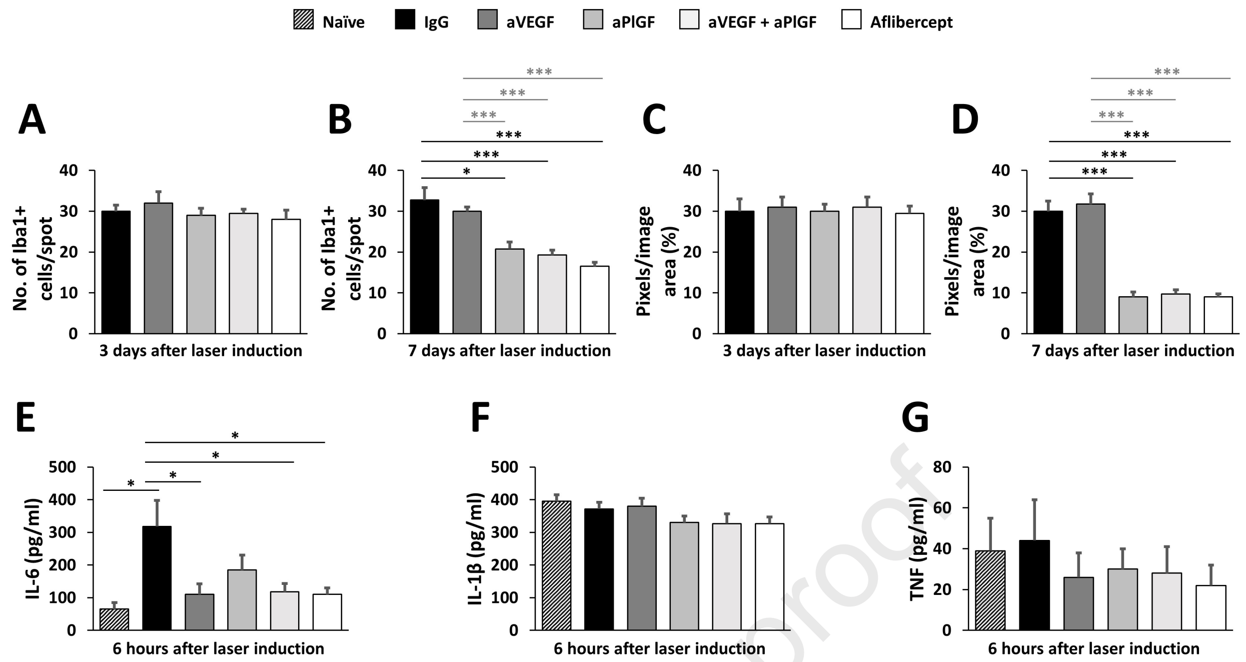

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