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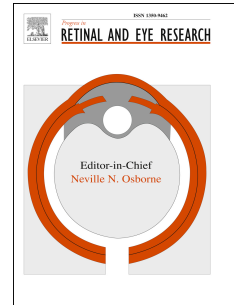
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# VEGFR1 signaling in retinal angiogenesis and microinflammation

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**Author contributions**

Percentage of work contributed by each author in the production of the manuscript is as follows:
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**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.

**VEGFR1 signaling in retinal angiogenesis and microinflammation**

**Abbreviations**

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

**Contents**

**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

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

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  ($VEGF-A_{xxxb}$) as well as the pro-angiogenic family ($VEGF-A_{xxxa}$) (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.

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.,

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*

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

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*  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.

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-1$\beta$, IL-6, IL-8, TNF-$\alpha$, 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

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

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

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**

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.

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**

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.

1031

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 | LUMINOUS – observational study |
| | | Multiple retinal disease indications | 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 observational studies |
| | | Multiple retinal disease indications | 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.

1038

**Figure legends**

Figures 1-4 are to be reproduced in color

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

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

**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.

1074  Reproduced under Creative Common CC-BY license (Apicella et al., 2018).

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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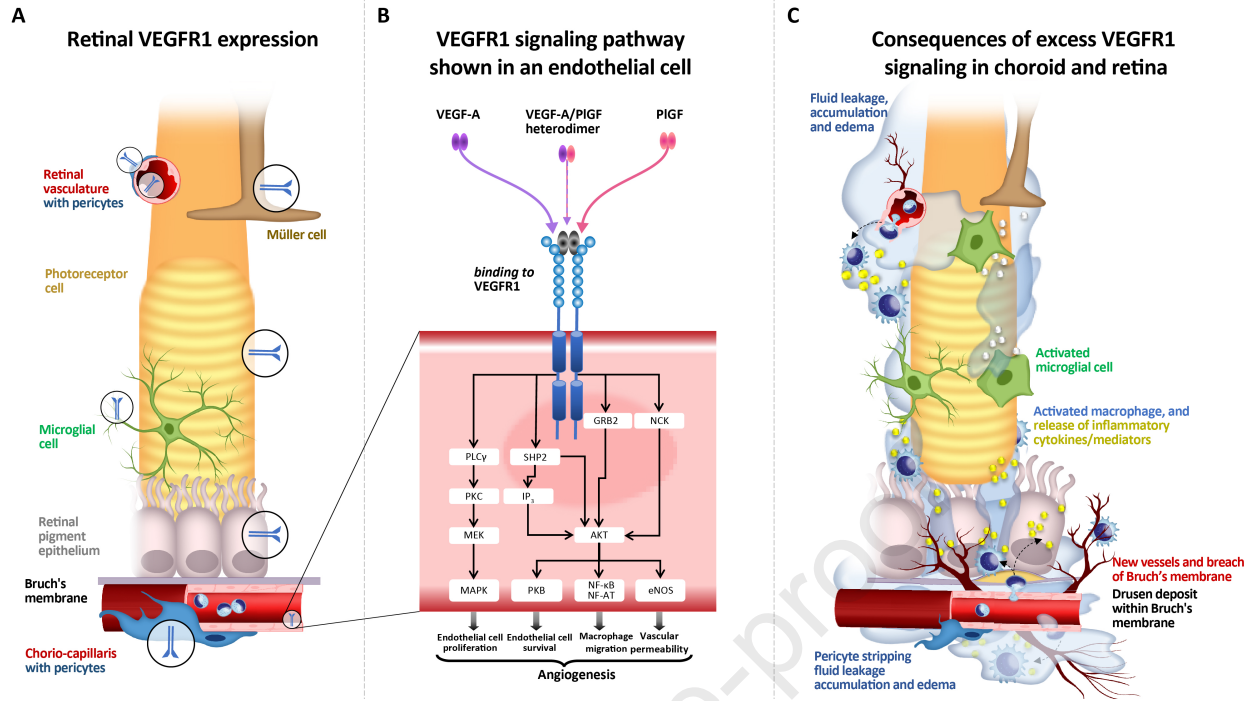2051 aflibercept treatment for retinopathy of prematurity. Graefes Arch Clin Exp Ophthalmol 257, 49-55.

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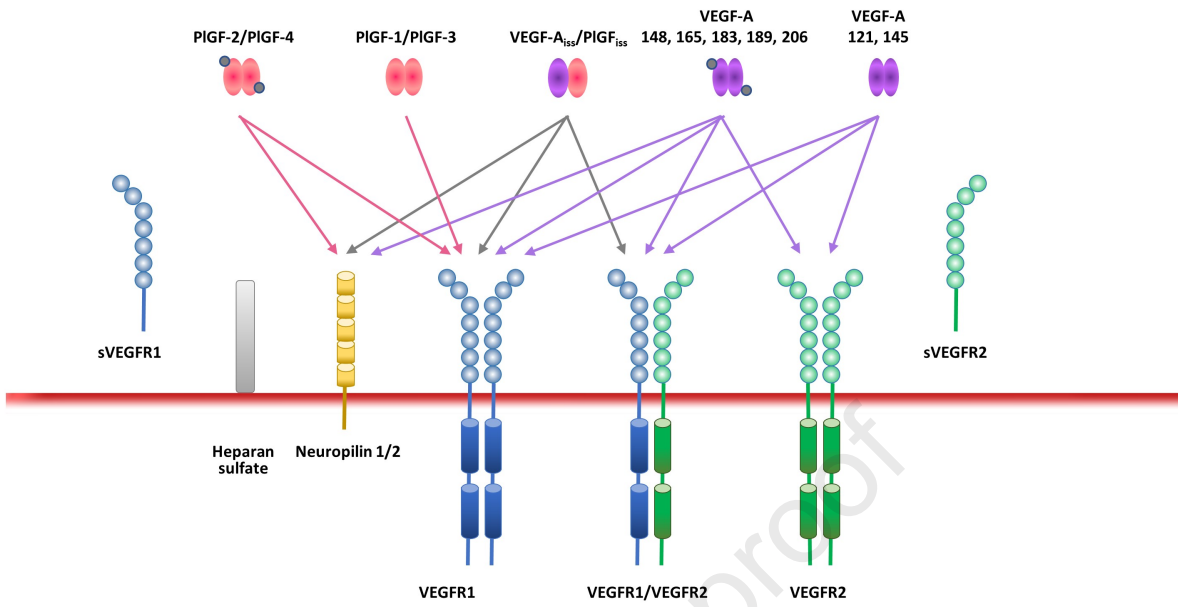
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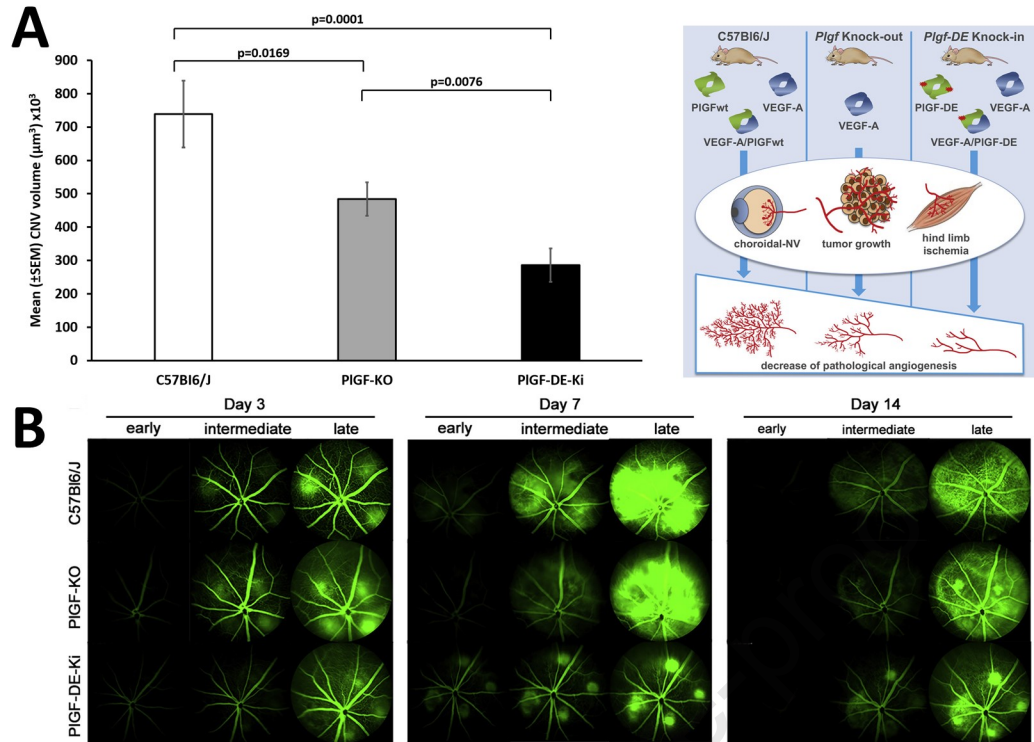
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**A** Retinal VEGFR1 expression

Retinal vasculature with pericytes

Müller cell

Photoreceptor cell

Microglial cell

Retinal pigment epithelium

Bruch's membrane

Chorio-capillaris with pericytes

**B** VEGFR1 signaling pathway shown in an endothelial cell

VEGF-A

VEGF-A/PlGF heterodimer

PlGF

binding to VEGFR1

GRB2    NCK

PLCγ    SHP2

PKC    IP₃

MEK    AKT

MAPK    PKB    NF-κB NF-AT    eNOS

Endothelial cell proliferation    Endothelial cell survival    Macrophage migration    Vascular permeability

Angiogenesis

**C** Consequences of excess VEGFR1 signaling in choroid and retina

Fluid leakage, accumulation and edema

Activated microglial cell

Activated macrophage, and release of inflammatory cytokines/mediators

New vessels and breach of Bruch's membrane

Drusen deposit within Bruch's membrane

Pericyte stripping fluid leakage accumulation and edema

PlGF-2/PlGF-4    PlGF-1/PlGF-3    VEGF-A$_{iss}$/PlGF$_{iss}$    VEGF-A
148, 165, 183, 189, 206    VEGF-A
121, 145

sVEGFR1    sVEGFR2

Heparan
sulfate    Neuropilin 1/2

VEGFR1    VEGFR1/VEGFR2    VEGFR2

**A**

**B**

| | Day 3 | Day 7 | Day 14 |
| --- | --- | --- | --- |
| | early  intermediate  late | early  intermediate  late | early  intermediate  late |

**A** CD31 NG2

Control

APB5

**B** HE

Control

APB5

**C**

CD45$^{lo}$ CD11b$^+$ | CD45$^{hi}$ CD11b$^+$

Control

| 25% | 12% | 15.6% | 75.6% |
| 58% | 5% | 0% | 8.89% |

APB5

| 25.5% | 14.4% | 11.1% | 80.1% |
| 47.3% | 12.8% | 1.21% | 7.63% |

VEGFR1 (y-axis)  Ly6C (x-axis)

**D** *Vegfa*  *Pgf*

Control

APB5

Type IV collagen  Iba1

**E** VEGFR1 Iba1

Control

APB5

**F** *VEGFR1-TK$^{+/+}$*  *VEGFR1-TK$^{-/-}$*

Retinal cup

CD31 Iba1

Iba1$^+$ cell no./area

Vessel density (%)

□ *VEGFR1-TK$^{+/+}$*  ■ *VEGFR1-TK$^{-/-}$*

**G** IgG  Aflibercept

Pre

Post

(µm)

Cell movement (µm/h)

NS  ***

Dendrite length (µm)

NS  ***

IgG  Aflibercept

■ Pre  ■ Post

**H** IgG  Aflibercept

IB4 ICAM-1

Iba1

**I**

VEGF-A
PlGF  VEGFR1  Microglia

VEGF-A  MP  Monocyte

VEGFR2  CCR2

EC  NFAT  CCL2

Naïve    IgG    aVEGF    aPlGF    aVEGF + aPlGF    Aflibercept

**A** 3 days after laser induction — No. of Iba1+ cells/spot

**B** 7 days after laser induction — No. of Iba1+ cells/spot

**C** 3 days after laser induction — Pixels/image area (%)

**D** 7 days after laser induction — Pixels/image area (%)

**E** 6 hours after laser induction — IL-6 (pg/ml)

**F** 6 hours after laser induction — IL-1β (pg/ml)

**G** 6 hours after laser induction — TNF (pg/ml)

Data are mean (±SEM); *p<0.05; **p<0.01; ***p<0.001