PlGF and VEGF-A/PlGF Heterodimer are Crucial for Recruitment and Activation of Immune Cells During Choroid Neovascularization

Valeria Tarallo,[1] Sara Magliacane Trotta,[1] Sonia Panico,[1] Luca D'Orsi,[1,2] Grazia Mercadante,[1] Valeria Cicatiello,[1] and Sandro De Falco[1–3]

[1]Angiogenesis Lab, Institute of Genetics and Biophysics 'Adriano Buzzati-Traverso' - CNR, Naples, Italy
[2]BIOVIIIx srl, Via Alessandro Manzoni 1, Napoli, Italy
[3]AnBition srl, Via Alessandro Manzoni 1, Napoli, Italy

Correspondence: Sandro De Falco and Valeria Tarallo, Institute of Genetics and Biophysics "Adriano Buzzati Traverso," CNR, Via Pietro Castellino, 111, Naples 80131, Italy; sandro.defalco@igb.cnr.it; valeria.tarallo@igb.cnr.it.

VT and SDF contributed equally to this work.

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Purpose. Recruitment and activation of inflammatory cells, such as retinal microglia/macrophages, in the subretinal space contribute significantly to the pathogenesis of age-related macular degeneration (AMD). This study aims to explore the functional role of vascular endothelial growth factor (VEGF-A), placental growth factor (PlGF) and VEGF-A/PlGF heterodimer in immune homeostasis and activation during pathological laser-induced choroidal neovascularization (CNV).

Methods. To investigate these roles, we utilized the PlGF-DE knockin (KI) mouse model, which is the full functional knockout (KO) of PlGF. In this model, mice express a variant of PlGF, named PlGF-DE, that is unable to bind and activate VEGFR-1 but can still form heterodimer with VEGF-A.

Results. Our findings demonstrate that, although there is no difference in healthy conditions, PlGF-DE-KI mice exhibit decreased microglia reactivity and reduced recruitment of both microglia and monocyte-macrophages, compared to wild-type mice during laser-induced CNV. This impairment is associated with a reduction in VEGF receptor 1 (VEGFR-1) phosphorylation in the retinae of PlGF-DE-KI mice compared to C57Bl6/J mice. Corroborating these data, intravitreal delivery of PlGF or VEGF-A/PlGF heterodimer in PlGF-DE-KI mice rescued the immune cell response at the early phase of CNV compared to VEGF-A delivery.

Conclusions. In summary, our study suggests that targeting PlGF and the VEGF-A/PlGF heterodimer, thereby preventing VEGFR-1 activation, could represent a potential therapeutic approach for the management of inflammatory processes in diseases such as AMD.

Keywords: VEGF family, inflammation, choroidal neovascularization (CNV), microglia, monocyte-macrophages, age-related macular degeneration (AMD)

Pathological ocular neovascularization, characterized by abnormal blood vessel growth in the chorioretinal area, is a leading cause of blindness associated with eye diseases, such as age-related macular degeneration (AMD), diabetic retinopathy (DR), retinal vein occlusion (RVO), and retinopathy of prematurity (ROP). There are two main types of ocular neovascularization: retinal neovascularization and choroidal neovascularization (CNV).[1] Retinal neovascularization is typically induced by hypoxia and is linked to DR, RVO, and ROP. CNV, on the other hand, results from abnormalities in Bruch's membrane and the retinal pigment epithelium (RPE) and is responsible for irreversible vision loss in the majority of AMD cases.[2]

Studies have demonstrated that microglia, the resident myeloid-derived cells in the retina, play a role in the development and progression of CNV.[3] In a healthy retina, microglia act as a surveillance network in the plexiform layers to maintain tissue integrity.[4] However, following injury, these inflammatory cells become activated and migrate to the damaged retinal site. They aid in the clearance of dying retinal cells, facilitate tissue repair, and release pro-inflammatory molecules.[5] This, in turn, recruits and activates additional immune cells, intensifying the inflammatory response. Chronic inflammation can lead to retinal tissue damage, including the macula, and contribute to disease development.[6,7]

For pathological ocular neovascularization to occur, pro-angiogenic members of vascular endothelial growth factor (VEGF) family are required. Among them, VEGF-A plays a fundamental role in activating endothelial cells and stimulating the formation of new blood vessels through its specific receptor VEGFR-2.[8–10] Placental growth factor (PlGF) has a dual role. It also acts on endothelial cells, but several reports have demonstrated its ability to recruit and activate inflammatory cells in various pathological contexts, such as tumor growth, rheumatoid arthritis, and CNV, representing a

link between angiogenesis and inflammation.[11–13] This ability is exerted through the activation of its specific receptor, VEGFR-1 (also known as Flt-1 in mice), although this receptor is also recognized by VEGF-A. Unlike VEGFR-2, VEGFR-1 is expressed in inflammatory cells, such as monocyte-macrophages, dendritic cells, and retinal microglia, and also in many other cell types as bone marrow stem/precursor cells, endothelial cells, stromal cells, pericytes, RPE, and photoreceptors. When activated by PlGF, VEGFR-1 induces mainly survival, proliferation, and migration pathways.[14]

VEGF-A and PlGF act synergistically not only because they share VEGFR-1 as a common receptor but also because, when co-expressed in the same cells, they can form heterodimer.[15] VEGF-A/PlGF can bind and activate VEGFR-1 and, similar to VEGF-A, can also bind VEGFR-1/VEGFR-2 heterodimer.[12] Among these two ligands and the two receptors, the only knockout that allow survival during embryonic development is the knockout of PlGF. In adult PlGF-knockout (KO) mice, pathological neovascularization required in diseases models, such as cancer growth and CNV, is strongly impaired, indicating that the role of PlGF is confined to pathological angiogenesis.[9,16]

We recently reported the generation of the full functional knockout of PlGF achieved by knockin (KI) of a variant of PlGF named PlGF-DE.[12,17] PlGF-DE is unable to bind and activate VEGFR-1 but can still form heterodimer with VEGF-A. Therefore, unlike PlGF-KO, where both PlGF and the heterodimer are absent, in PlGF-DE-KI mice, both are present but non-functional.[12,18] In this mouse model, pathological angiogenesis associated with cancer growth, CNV and hind limb ischemia is even more impaired compared to PlGF-KO.[18] The additional angiogenesis inhibition observed in PlGF-DE-KI mice is due to the capability of PlGF-DE monomers to form inactive VEGF-A/PlGF-DE heterodimers acting as dominant negative of VEGF-A.

The PlGF-DE-KI mouse model represents a new tool for dissecting the functional role of VEGF-A, PlGF, and VEGF-A/PlGF heterodimer in immune homeostasis and immune cells activation during pathological conditions. In this study, we decided to characterize retinal immune cells in PlGF-DE-KI, PlGF-KO, and wild-type (C57Bl6/J background) mice under physiological condition and in a laser induced CNV model, an established system for studying key aspects of neovascular AMD.[19] Our results demonstrate that although there is no difference in healthy conditions, the recruitment and activation of immune cells, particularly microglia, are significantly impaired in PlGF-DE-KI mice compared to wild-type mice. These data highlight the pivotal role of VEGFR-1 signaling and its ligands PlGF and VEGF-A/PlGF in immune cell activation, leading to a reduction in inflammatory events and the development of CNV lesions.

Methods

Animals

The C57Bl6/J mice were purchased from Charles River. Animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Animal Statement for the Use of Animals in Ophthalmic and Vision Research, with the European directives no. 2010/63/UE and Italian directives D.L. 26/2014, and were approved by the Italian Ministry of Health (authorization no. 30/2023-PR of 23/01/2023). For all procedures, 6- to 8-week-old mice were used, and anesthesia was performed by intraperitoneal injection of 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine.

Choroidal NeoVascularization Model

Laser photocoagulation was performed as previously described.[18] For experiments of CNV stimulation in PlGF-DE-KI mice, 0.2 pmol in 1 µL of PBS of recombinant VEGF-A, or VEGF-A/PlGF heterodimer (R&D Systems), or PlGF was intravitreally injected in PlGF-DE-KI mice immediately after the laser injury, with a microsyringe (Hamilton) carrying a 33G needle. As control, 1 µL of PBS was delivered to the contralateral eyes. Three or 7 days after laser injury, the eyes were enucleated and processed for immunostaining analysis.

Flow Cytometry

Retinas from C57Bl6/J and PlGF-DE-KI mice were used for flow cytometry analysis at day 3 after the laser application. The eyes were collected in PBS (pH 7.4), the anterior segment was removed, and the retina was mechanically detached from the choroid-RPE complex. Retinas of each individual mouse were analyzed as one sample. Retinas were processed according to the guidelines of the Neural Tissue Dissociation Kit for postnatal neurons (MACS; Miltenyi Biotec). Single-cell suspensions were obtained and stained with Viobility 405/452 Fixable Dye (Miltenyi Biotec) for detection of dead cells. The samples were washed in DPBS and resuspended in FACS buffer (2 mM EDTA, 0.5% BSA, in DPBS). Samples were incubated for 20 minutes with an FcR Blocking Reagent (1:200; Miltenyi Biotec), followed by incubation with the fluorescent-labeled antibodies for 20 more minutes at 4°C in the dark. Dissociated retinas were stained with CD11b-phycoerythrin (clone M1/70; 1:200, Miltenyi Biotec) and CD45.2-allophycocyanin (clone 104-2; 1:200; Miltenyi Biotec). After the completion of staining, the cells were washed and rinsed in DPBS. Data were acquired with FACSCanto (BD Biosciences). Raw flow cytometry data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR, USA). Each experiment was repeated five times.

Statistical Analyses

Experiments were all performed with a minimum of three biological samples. Replicate number is indicated in the figure legends as n = x. Results are presented as the mean ± standard error of the mean (SEM). To determine the significance between the two groups, comparisons were made using the Student's t test with P values < 0.05 considered statistically significant.

Results

Characterization of Immune Cells in PlGF-DE-KI Mice in Physiological Condition

We previously demonstrated that PlGF-DE-KI mice had a more significant impairment in angiogenesis associated with tumor growth, hindlimb ischemia, and CNV when compared to both PlGF-KO and wild-type mice (C57Bl6/J background).[18] To investigate whether this change was due to defects or alterations in immune cells, we decided to analyze and phenotype these cells in PlGF-DE-KI mice using flow cytometry analysis. Specifically, we examined T cells, B cells, natural killer cells, dendritic cells, Treg

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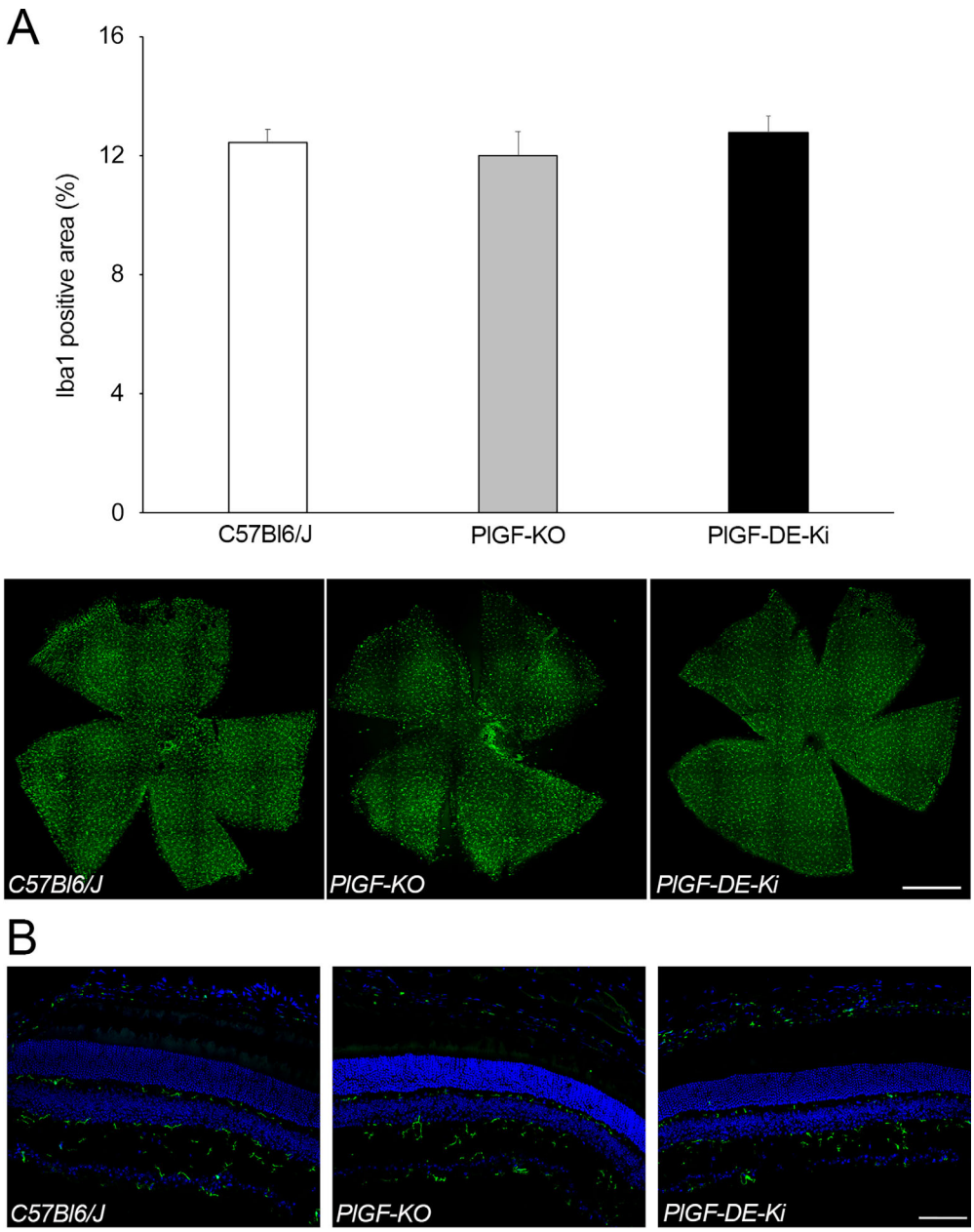

Figure 1. Immune cells distribution in retinae of C57Bl6/J, PlGF-KO, and PlGF-DE-KI mice. (**A**) Iba1 positive area were measured in staining performed on retina flat mount of C57Bl6/J, PlGF-KO, and PlGF-DE-KI ($n = 6$ retinae per group). Data are presented as the mean ± SEM. No significative difference was observed among the three groups. On the bottom are representative pictures of Iba1 staining. Scale bar = 1000 µm. (**B**) Representative images of Iba1 immunostaining on retinal sections. Nuclei are counterstained with 4′,6-diamidino-2-phenylindole (DAPI; *blue*). Scale bar = 100 µm.

cells, myeloid cells and macrophage populations from bone marrow, lymph nodes, and spleen (Supplementary Methods, Table S1). As shown in Supplementary Figure S1, we did not observe any significant alterations in these specific immune cells between C57Bl6/J mice and PlGF-DE-KI mice. Because PlGF-DE-KI mice exhibited a more severe phenotype compared to PlGF-KO mice, we did not perform immunophenotype analysis for these mice.

Considering the immune privilege of the eye and the significant impairment that we observed in PlGF-DE mutant mice compared to both PlGF-KO and C57Bl6/J mice after laser insults,[18] we decided to focus our attention on the immune cells of the retina. Therefore, we performed an immunostaining analysis on retinal flat-mounts using the ionized calcium binding adaptor molecule 1 (Iba1) antibody, that recognize retinal microglia and macrophages (see Supplementary Methods). This allowed us to identify and characterize the distribution and morphology of resident retinal microglia. As showed in Figure 1A, we did not observe any alterations in distribution and amount of $Iba1^+$ cells in C57Bl6/J, PlGF-KO, and PlGF-DE-KI mice. This observation was also confirmed by immunohistochemical analyses with Iba1 in retinal cross sections shown in Figure 1B. In lane with these results, there were no signif-

icant differences in the expression of *Vegf-a*, *PlGF*, *Vegfr-1*, and *Vegfr-2* in the retina of C57Bl6/J, PlGF-KO, and PlGF-DE-KI mice (Supplementary Fig. S2).

Collectively, these data indicate that PlGF-DE mutant mice do not exhibit any alterations of immune cell phenotype, distribution, and amount of resident retinal microglia in physiological conditions.

PlGF-DE-KI Mice Show Impaired Immune Cells Recruitment After Laser Photocoagulation

To investigate whether the impaired pathological angiogenesis observed in PlGF-DE-KI mice with respect to wild-type mice after laser-induced CNV was due to immunomodulatory defects, we performed laser photocoagulation in these mice in order to characterize the immune-related effects. Therefore, we performed immunostaining analysis on retinal cryosections using Iba1 antibody after 3 and 7 days from laser insult (see Supplementary Methods). At day 3, $Iba1^+$ cells were largely present in the CNV area, and some cells were also distributed in the other layers of retina in C57Bl6/J (Fig. 2A), whereas, in PlGF-DE-KI mice, $Iba1^+$ cells were also present in the CNV area and sparser and sporadically distributed in the other retinal layers (see Fig. 2A). At 7 days from laser insult, the immunoreactivity was similar in the two mice strain. In order to quantify the Iba1 signal, we performed immunofluorescence analyses on both the retina and the RPE/choroid tissues (see Supplementary Methods). Confocal images of the retinal flat mounts from C57Bl6/J mice revealed massive accumulation of reactive ameboid-shaped Iba^+ cells at the lesion site 3 days post-laser injury, whereas retinas from PlGF-DE-KI mice had less Iba1 phagocytes and these cells showed mainly a ramified morphology (Fig. 2B). The Iba^+ positive area within the retinas is significantly low in PlGF-DE mice as compared to control C57Bl6/J retinas (Fig. 2C). The infiltration of immune cells in the retina 7 days after laser injury was overall less than at 3 days, indicating a wound healing process (see Fig. 2B). In order to explore the difference between the two mice strain on subretinal and RPE-associated phagocytes, $Iba1^+$ cells were imaged in RPE/choroidal flat mounts (Fig. 2D). Similarly, as in the retina, at day 3, the PlGF-DE-KI mice showed a significant reduction of reactivity in the RPE/choroid compared to wild-type, whereas at day 7 we did not observe any significant differences (Fig. 2E).

Collectively these data demonstrate that PlGF-DE-KI mice show a reduced immune cells recruitment after laser damage.

Retinal Mononuclear Phagocytosis Activation and Monocyte Recruitment is Impaired in PlGF-DE-KI

Retinal mononuclear phagocyte reactivity has been shown to be crucial for the development of CNV.[20] In the healthy retina, microglia exhibit a distinct ramified morphology, whereas during the early inflammatory phase following laser insult, they are rapidly recruited to the lesion site and switched into amoeboid phagocytes defined as the activated phenotype. To determine the cellular phenotype at the lesion sites in PlGF-DE-KI mice, we assessed changes in microglial ramification by counting the number of crossing points per individual cell using a grid image system[21] on confocal images of retinal flat mount stained for Iba1 (see Fig. 2B). These analyses showed a significant increase in grid cross points in PlGF-DE-KI retinas compared to control C57Bl6/J retinas, which clearly indicates less activation of retinal microglial cells (Fig. 2F).

To specifically detect mononuclear phagocytes, we performed flow cytometry analysis at day 3 after CNV in the retina of PlGF-DE-KI mice, and as control of C57Bl6/J mice (Fig. 3A; Supplementary Fig. S3). Quantification of different cell populations revealed that there was no difference of $CD45^{low}Cd11b^+$ microglia and $CD45^{hi}CD11b^+$ macrophages in naïve retina of C57Bl6/J and PlGF-DE-KI mice. As expected, CNV induced accumulation of $CD45^{low}Cd11b^+$ microglia and $CD45^{hi}CD11b^+$ macrophages in the retina of C57Bl6/J mice. In contrast, PlGF-DE-KI retina showed a significant reduction of their number (Figs. 3B, 3C), thus indicating that in these mice there is an impairment in the recruitment of inflammatory cell at the lesion site after 3 days of laser insult.

We decided to characterize microglia reactivity in lasered PlGF-DE-KI and C57Bl6/J retinas by analyzing the expression of microglia marker genes, namely CC-chemokine ligand 2 (*Ccl2*) and activated microglia whey acidic protein (*Amwap*[19]; see Supplementary Methods). *Amwap* was isolated from activated primary retinal microglia cells, and its overexpression has been shown to reduce the release proinflammatory cytokine expression, including CCL2.[22] As expected, we observed an increase in the expression of both markers over time after CNV in both C57Bl6/J and PlGF-DE-KI retinas as compared to naïve retinas (Figs. 3D, 3E), indicating the presence of activated microglia. Interestingly, we found that in PlGF-DE-KI retinas, the increased expression of *Ccl2* was significantly lower than in C57Bl6/J, whereas the expression of *Amwap* was higher, thus confirming less microglia activation. We also evaluated retinal C-C chemokine receptor type 2 (*Ccr2*) expression because this receptor is only expressed in migrating inflammatory cells, but not in microglia.[23] As expected, we observed enhanced *Ccr2* mRNA expression after early time points of laser insult in both C57Bl6/J and PlGF-DE-KI retinas, as compared to naïve retinas (Fig. 3F), likely due to the accumulation of blood-derived $CCR2^+$ monocytes at the lesion sites in response to CCL2. Of note, we observed a significant decrease in *Ccr2* mRNA expression in PlGF-DE-KI, indicating impaired recruitment of blood-derived monocytes.

Collectively, these data demonstrated that PlGF-DE-KI mice showed an impaired recruitment of inflammatory cells (mononuclear phagocytes and monocyte-macrophages) after laser-induced CNV.

VEGFR-1 Activation is Impaired in PlGF-DE-KI Mice During Pathological Conditions

Because VEGFR-1 is expressed in immune cells and its activation is crucial for immune cells recruitment and activation and PlGF-DE-KI mice have both a PlGF-DE homodimer and VEGF-A/PlGF-DE heterodimer unable to bind and activate VEGFR-1, we expected to observe a reduced VEGFR-1 phosphorylation during pathological condition in PlGF-DE-KI mice compared with C57Bl6/J mice. To verify this hypothesis, we analyzed by Western blot (see Supplementary Methods) protein extracts from retinal tissues of C57Bl6/J and PlGF-DE-KI mice ($n = 4$ mice per group) collected at days 3 after laser-induced damage. As expected, VEGFR-1 phosphorylation increased at days 3 (+ 3.1 times) in wild-type

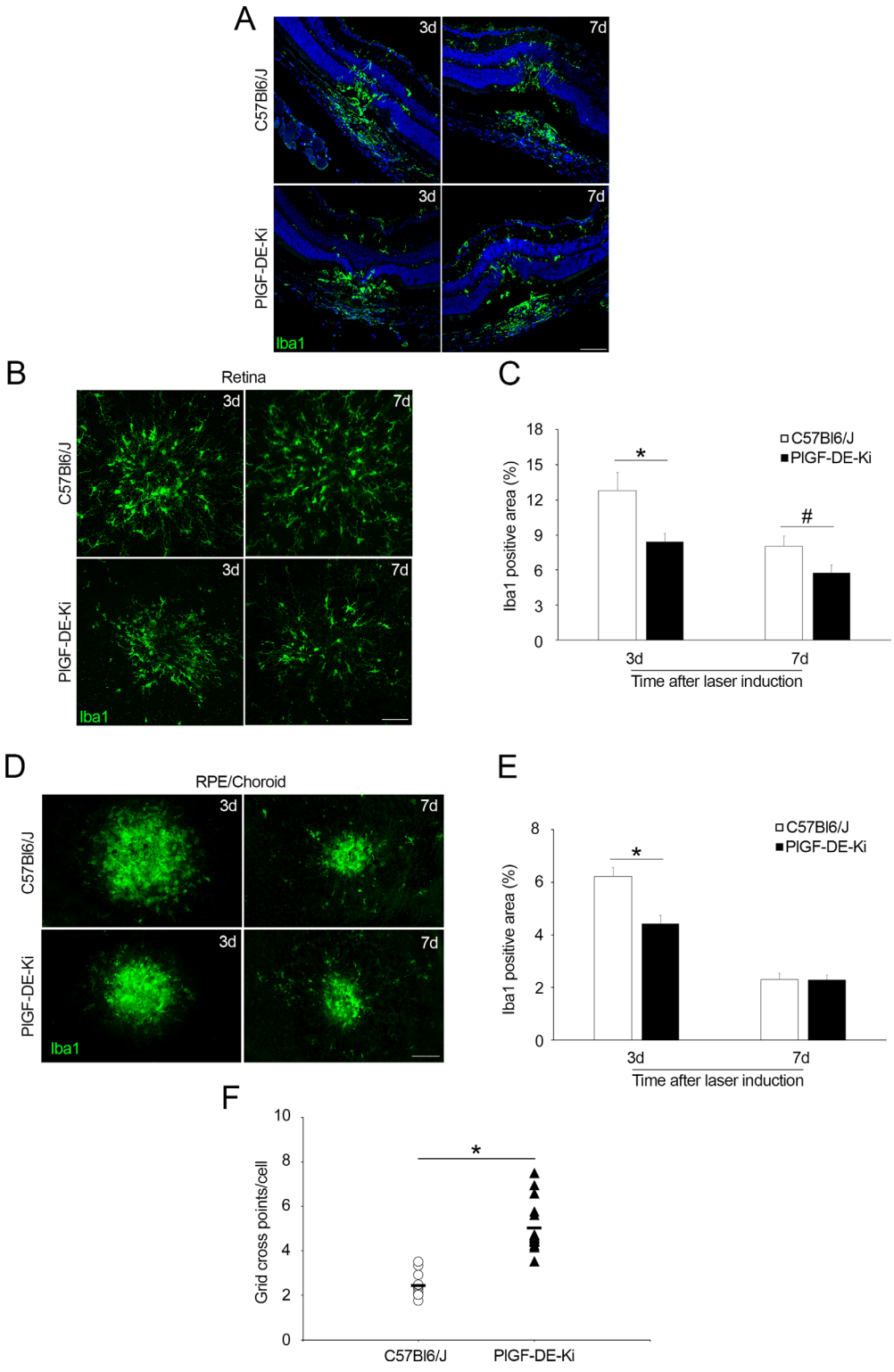

Figure 2. PlGF-DE-KI mice showed impaired immune cell recruitment after CNV. (**A**) Representative images of immunostaining of Iba1 on retinal sections after 3 and 7 days from laser injury in C57Bl6/J and PlGF-DE-KI mice. Nuclei are counterstained with DAPI, *blue*. Scale bar = 100 µm. (**B**) Representative images of Iba1$^+$ phagocytes on retinal flat mount after 3 and 7 days from laser injury in C57Bl6/J and PlGF-DE-KI mice. Scale bar = 50 µm. (**C**) Quantification of Iba1$^+$ area in lesions (n = 16/22 spots for each group). $^*P = 0.033$ and $^{\#}P = 0.047$. (**D**) Representative images of Iba1$^+$ cells on RPE/choroid flat mount after 3 and 7 days from laser injury in C57Bl6/J and PlGF-DE-KI mice. Scale bar = 100 µm. (**E**) Quantification of Iba1$^+$ area in lesions (n = 21/30 spots for each group). $^*P = 0.001$. (**F**) Quantification of Iba1$^+$ morphology in laser spots using a grid image analysis system (n = 16 spots for each group, and n = 15/20 cells for each spot). $^*P = 0.0001$. For all panels, data are presented as the mean ± SEM.

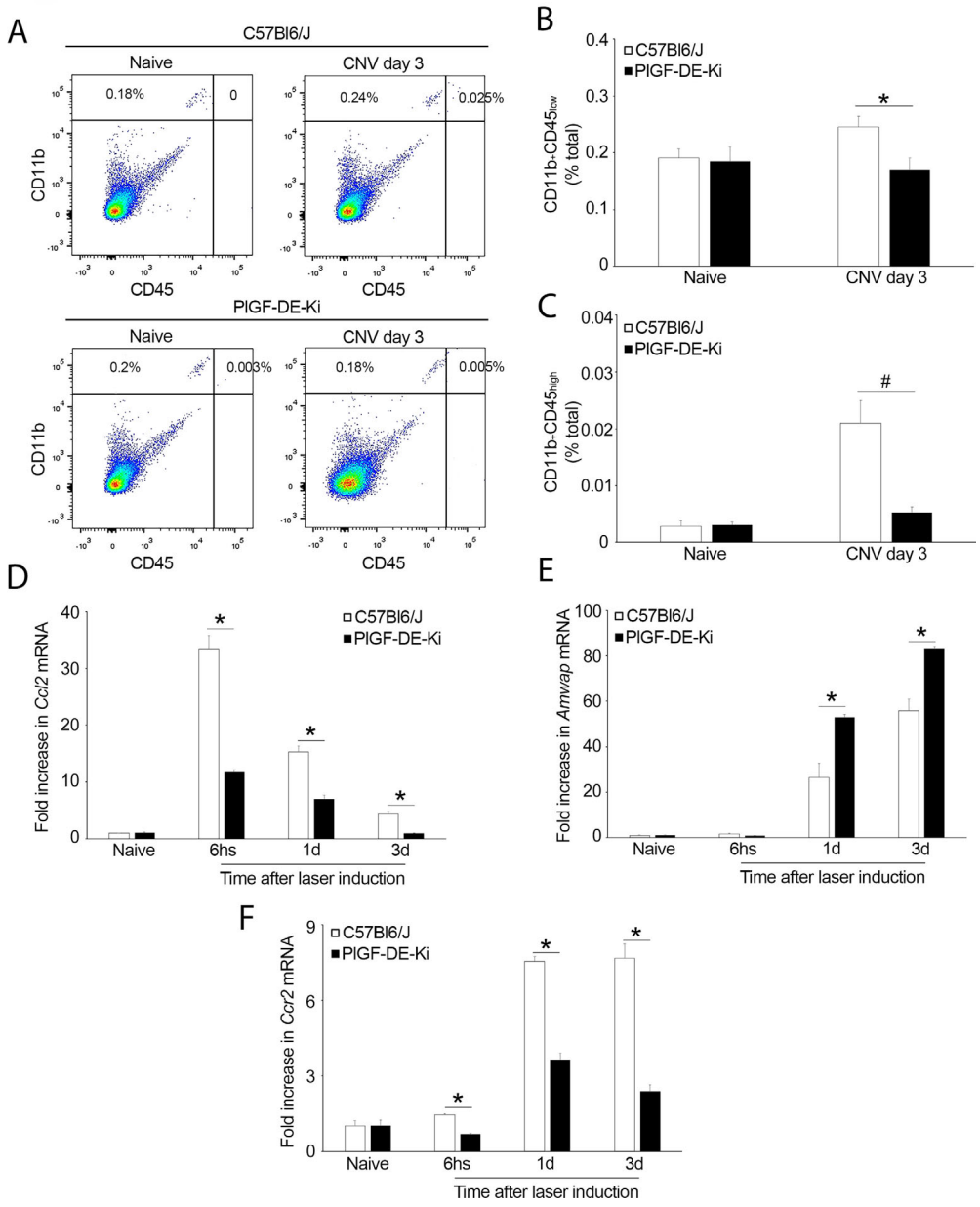

Figure 3. PlGF-DE-KI mice showed impaired recruitment of mononuclear phagocytes and monocyte-macrophages after laser-induced CNV. (**A**) Representative flow cytometry plots at day 3 after CNV in C57Bl6/J and PlGF-DE-KI mice. Microglia were identified as $CD45^{low}$ $CD11b^{+}$ and macrophages as $CD45^{hi}$ $CD11b^{+}$. (**B, C**) Quantification of microglia ($CD45^{low}$ $CD11b^{+}$; *top panel*) and macrophages ($CD45^{hi}$ $CD11b^{+}$; *bottom panel*) in the retina 3 days after CNV in C57Bl6/J and PlGF-DE-KI mice. A reduced number of microglia and macrophages is observed in PlGF-DE-KI compared to C57Bl6/J retinae ($n = 5$). $^{*}P = 0.05$ and $^{\#}P = 0.026$). Gating strategy and isotype control antibody are shown in Supplementary Figure S2. (**D, E**) The mRNA relative expression was evaluated by qRT-PCR using the $2^{-\Delta Ct}$ method normalized on *Gapdh* expression. Evaluation of *Cccl2* (**D**), *Amwap* (**E**), and *Ccr2* (**F**) mRNA levels in retinae of C57Bl6/J and PlGF-DE-KI mice ($n = 3$). $^{*}P < 0.05$. For all panels, data are presented as the mean ± SEM. See also Supplementary Figure S2.

mice compared with unlasered tissue (Control). Conversely, a minimal increase of VEGFR-1 phosphorylation at days 3 (+ 1.1 times) was observed in PlGF-DE-KI mice (Fig. 4A). Furthermore, we found that the level of VEGFR-1 phosphorylation in the unlasered tissue (Control) was noticeably reduced in PlGF-DE-KI mice compared to wild-type mice (see Fig. 4A). Interestingly, we did not observe any significant reduction in the levels of VEGFR1 phosphorylation under physiological conditions between PlGF-KO and PlGF-DE-KI mice (Supplementary Fig. S4). This finding suggests that the basal level of VEGFR-1 phosphorylation in C57Bl6/J mice in physiological condition is mainly exerted by PlGF homodimer and VEGF-A/PlGF heterodimer.

In addition, we also evaluated VEGFR-1 phosphorylation in the area of CNV lesion 3 days after laser damage immunolabeling retinal cryosections for phosphorylated VEGFR-1 (pVEGFR1) and F4/80, which detect both monocyte-macrophage and microglia cells (Fig. 4B, Supplementary Fig. S5 for isotype control antibody). Interestingly, in both PlGF-DE-KI and wild-type mice, the pVEGFR-1 signal co-localize with F4/80, thus indicating that VEGFR-1 signaling is required for immune cells recruitment and activation.

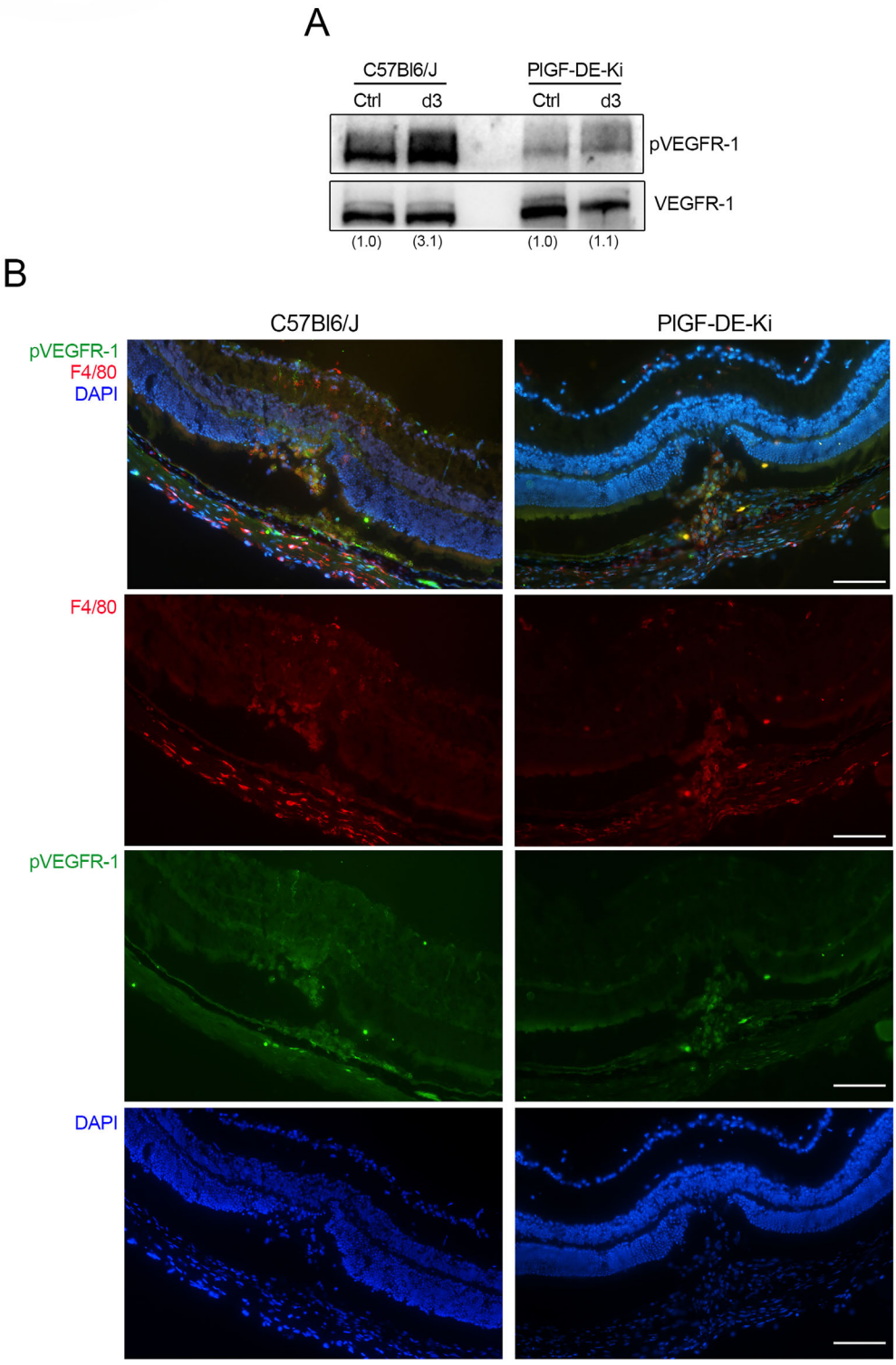

Figure 4. VEGFR-1 phosphorylation is reduced in retinae of PlGF-DE-KI mice in normal condition and during laser-induced CNV. (**A**) Western blot analysis of VEGFR-1 phosphorylation (pVEGFR-1) in retinae lysates from C57Bl6/J and PlGF-DE-KI mice ($n = 4$ per groups), harvested at day 3 after laser damage. As control, extracts from unlasered retinae were used (Control). Densitometry values normalized against VEGFR-1 (anti-VEGFR-1) are shown in *parentheses*. (**B**) Representative images of immunolocalization of phosphorylated VEGFR-1 and F4/80 on CNV lesion. Scale bars = 100 µm. See also Supplementary Figure S3.

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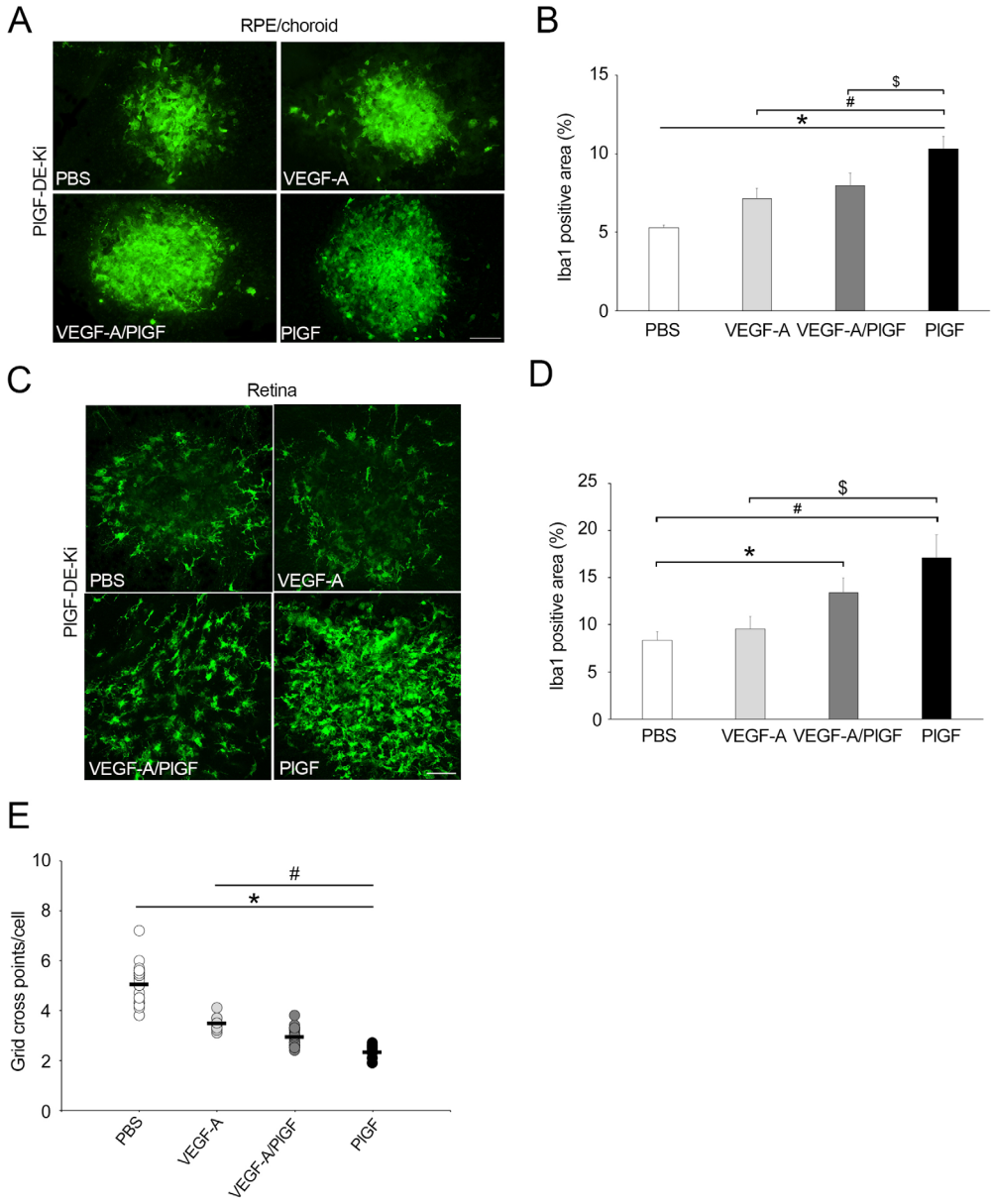

Figure 5. PlGF and VEGF-A/PlGF are the main drivers of immune cells recruitment and activation. (**A**) Representative images of Iba1$^+$ cells on RPE/choroid flat mount after 3 days from laser injury in PlGF-DE-KI mice intravitreally injected with PBS or 0.2 pM of VEGF-A, VEGF-A/PlGF, or PlGF. Scale bar = 100 µm. (**B**) Quantification of Iba1$^+$ area in lesions (n = 23/34 spots per group). *P < 0.05 vs. PBS, $^{\#}P$ = 0.03 vs. VEGF-A, and $^{\$}P$ = 0.042 vs. VEGF-A/PlGF. (**C**) Representative images of Iba1$^+$ phagocytes on retinal flat mount after 3 days from laser injury in PlGF-DE-KI mice intravitreally injected with PBS or 0.2 pM of VEGF-A, VEGF-A/PlGF, or PlGF. Scale bar = 50 µm. (**D**) Quantification of Iba1$^+$ area in lesions (n = 15/32 spots per group). *P = 0.002 and $^{\#}P$ = 0.0001 vs. PBS, and $^{\$}P$ = 0.006 vs. VEGF-A. (**E**) Quantification of Iba1$^+$ morphology in laser spots using a grid image analysis system (n = 15 spots per group, and n = 15/20 cells for each spot). *P = 0.0001 vs. PBS and $^{\#}P$ = 0.0001 vs. VEGF-A and VEGF-A/PlGF. For all panels, data are presented as the mean ± SEM.

Collectively, these findings demonstrate that the reduced inflammatory response observed in PlGF-DE-KI mice is due to decreased VEGFR-1 activation.

PlGF and VEGF/PlGF are the Main Drivers of Immune Response

In order to investigate which are the drivers of immune cell response during laser-induced CNV, we performed a rescue experiment in PlGF-DE-KI mice by intravitreally injecting VEGFR-1 ligands. To this purpose, 0.2 pmol recombinant heterodimer, or an equimolar amount of PlGF and VEGF-A, and as control PBS, were intravitreally administered in PlGF-DE-KI mice after the laser burns. At day 3, the immune cell response was evaluated through immunostaining analyses using the Iba1 antibody. Immunostaining of RPE/choroidal flat mounts revealed a significant increase of RPE-associated phagocytes in mice injected with PlGF, VEGF-A/PlGF, or VEGF-A compared to those injected with PBS. On note, in mice injected with PlGF the increase of Iba1 positive area was significant compared to VEGF-A/PlGF or VEGF-A injected mice (Figs. 5A, 5B). Confocal images of retinal flat mounts from PBS and VEGF-A injected mice revealed

accumulation of reactive Iba$^+$ cells at the lesion that exhibit mainly a ramified morphology. In the retinae of PlGF-DE-KI mice injected with VEGF-A/PlGF and PlGF, the Iba1$^+$ cells increased, and the latter displayed a more amoeboid shape (Fig. 5C). The Iba positive area indicated that this accumulation was significantly higher in VEGF-A/PlGF and PlGF injected mice compared to PBS, whereas no significative differences were observed in VEGF-A injected mice (Fig. 5D). Morphological analysis demonstrated that the three ligands rescue phagocyte reactivity of PlGF-DE mice with different degree of activity, with a greater increase of ameboid cells observed in mice injected with PlGF and a lower one in those injected with VEGF-A, as compared to the controls (Fig. 5E).

Taken together, these data provide compelling evidence that PlGF and the heterodimer play a crucial role as mediators of immune cells recruitment during CNV.

Discussion

Recruitment and accumulation of inflammatory cell populations, such as retinal microglia/macrophages, in the subretinal space contribute significantly to the pathogenesis of AMD.[21] Understanding the mechanisms that regulate this recruitment could be crucial for the design of therapeutic strategies targeting microglia/ macrophage chemotaxis and activation in AMD.

The current treatment for neovascular AMD relies on intravitreal injection of VEGF inhibitors.[24] However, blocking VEGF-A alone does not prevent the accumulation of reactive microglia and macrophages in laser lesions.[25] Among VEGF-A inhibitors, Aflibercept, a fusion protein consisting of the ligand-biding elements of VEGFR-1 and VEGFR-2, acts as a decoy receptor binding not only to VEGF-A, but also to VEGF-B and PlGF.[26] Aflibercept has been shown to negatively affect CNV by also reducing mononuclear phagocytes accumulation in the lesion area.[27] In line with these results, by using specific neutralizing antibodies, the block of VEGFR-2 does not inhibit phagocytes accumulation while it occurs by inhibiting VEGFR-1 activation in the early stage of CNV. Moreover, it has been demonstrated that VEGFR-1 and VEGFR-2 have differential roles in the development of CNV, with VEGFR-1 playing a dominant role in the early phase after laser, whereas blocking both receptors is necessary at the later phases of laser damage.[28] This differential role may reflect the spatiotemporal distribution of myeloid cell subsets during CNV formation, where microglia cells constitute a major cell population in the diseased retina,[7] further suggesting the importance of VEGFR1 signaling in the early phase of CNV.

For a long time, macrophage-derived VEGF-A was believed to be an important factor for inflammation-driven angiogenesis in CNV.[29,30] However, a recent study using several myeloid-specific VEGF-A KO models has shown that myeloid-derived VEGF-A production does not contribute significantly to CNV and vascular leakage.[31] This controversy has reinforced the suggestions that PlGF, rather than VEGF-A, could be responsible of inflammation during CNV. Indeed, a recent report has demonstrated that the PlGF/VEGFR1 axis is important in the early phase of CNV as its inhibition reduces the accumulation of mononuclear phagocytes.[27]

In line with these findings, our study definitively suggests the central role of PlGF in early CNV and introduces the VEGF-A/PlGF heterodimer as another candidate in the context of inflammation and CNV progression.

The PlGF-DE-KI mouse model has served as a valuable genetic tool in our investigation to dissect the functional roles of VEGF-A, PlGF, and VEGF-A/PlGF heterodimer in immune homeostasis and immune cells activation during pathological laser-induced CNV. In this mouse model, pathological angiogenesis associated with cancer growth, CNV, and hind limb ischemia, is even more impaired compared to PlGF-KO.[18] Once again, we confirm that both PlGF and heterodimer are redundant in physiological conditions, as we do not observe any alterations in the immunophenotype or distribution of immune retinal cells in these mice compared to wild-type mice (see Fig. 1, Supplementary Fig. S1). However, this situation changes in pathological conditions. After laser photocoagulation, we demonstrate that PlGF-DE-Ki mice exhibit impaired recruitment of Iba$^+$ cells (see Fig. 2). Specifically, these mice show a decrease in both microglia reactivity and recruitment, as well as in monocyte-macrophages recruitment (see Fig. 3). Both of these immune cell populations have been shown to be essential in the progression of CNV.[32,33]

Taking into consideration the importance of the VEGFR-1 receptor in immune cell activation and recruitment,[14] it is crucial to acknowledge that the immunomodulatory defect observed in PlGF-DE-KI mice is not solely attributed to the presence of non-functional PlGF and heterodimers. It is also influenced by the capability of PlGF-DE to act as a dominant negative of VEGF-A, leading to a reduction in its levels. In laser-induced CNV, we observe a reduction of VEGFR-1 phosphorylation in the retinae of PlGF-DE-KI mice compared to C57Bl6/J mice (see Fig. 4). Notably, even in non-lasered tissue, the level of VEGFR-1 phosphorylation is significantly reduced in PlGF-DE-KI mice compared to wild-type mice, despite the presence of free VEGF-A. This indicates that basal level of VEGFR-1 phosphorylation in physiological condition is primarily exerted by PlGF homodimer and VEGF-A/PlGF heterodimer.

Therefore, our in vivo data clearly demonstrate the essential role of PlGF and VEGF-A/PlGF heterodimer in the immune cell response during laser-induced CNV. These results were further supported by rescue experiments in the same experimental model. Intravitreal delivery of PlGF or the recombinant heterodimer in PlGF-DE-KI mice rescue the immune cell response at the early phase of CNV compared to VEGF-A delivery (see Fig. 5), further highlighting the significance of VEGFR-1 in pathological contexts. While PlGF specifically recognizes VEGFR-1, the heterodimer is able to induce VEGFR-1 dimerization or VEGFR-1/VEGFR-2 heterodimerization, but not VEGFR-2 dimerization. This shifts the balance of VEGF receptor activation toward VEGFR-1, which is sufficient to stimulate immune response in vivo, confirming the significance of VEGFR1 activation in immune cells recruitment. Notably, our study confirms that the heterodimer not only plays an active role in angiogenesis process, promoting the formation of new vessel formation,[18] but also to significantly contributes to the immune response, which is crucial for the early phase of neo-angiogenesis.

In summary, this study provides valuable insights into the role of PlGF, the VEGF-A/PlGF heterodimer and VEGFR-1 in immune cell recruitment during CNV. These findings suggest that targeting the PlGF/VEGFR-1 axis and the VEGF-A/PlGF heterodimer could be potential therapeutic approaches for managing CNV and related inflammatory processes in diseases such as AMD.

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