Retinal VEGF-A Overexpression Is Not Sufficient to Induce Lymphangiogenesis Regardless of VEGF-C Upregulation and Lyve1$^+$ Macrophage Infiltration

Iori Wada,[1] Shintaro Nakao,[1,2] Muneo Yamaguchi,[1] Yoshihiro Kaizu,[1] Mitsuru Arima,[1] Shinichiro Sawa,[3] and Koh-Hei Sonoda[1]

[1]Department of Ophthalmology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
[2]Department of Ophthalmology, National Hospital Organization, Kyushu Medical Center, Fukuoka, Japan
[3]Division of Mucosal Immunology, Research Center for Systems Immunology, Kyushu University, Fukuoka, Japan

PURPOSE. No lymphatic vessels have been identified in the retina. This study investigated whether pathological VEGF-A–overexpressing diabetic retina causes lymphangiogenesis.

METHODS. Three genetic mouse models of diabetic retinopathy (DR) (Akita [*Ins2*$^{+/-}$], Kimba [*vegfa*$^{+/+}$], and Akimba [Akita × Kimba] mice) were used. Retinas were examined by fundus photography, fluorescence angiography (FA), and immunostaining to detect lymphangiogenesis or angiogenesis. Lyve1-GFP (*Lyve1$^{EGFP/Cre}$*) mice were used to examine Lyve1-expressing cells by immunostaining. Lymphatic-related factors were investigated in mouse retina and vitreous fluid from proliferative diabetic retinopathy (PDR) patients by RT-PCR and ELISA, respectively. Aged Kimba and Akimba mice were used to examine the retinal phenotype at the late phase of VEGF overexpression.

RESULTS. FA and immunostaining showed retinal neovascularization in Kimba and Akimba mice but not wild-type and Akita mice. Immunohistochemistry showed that lymphangiogenesis was not present in the retinas of Akita, Kimba, or Akimba mice despite the significant upregulation of lymphatic-related factors (Lyve1, podoplanin, VEGF-A, VEGF-C, VEGF-D, VEGFR2, and VEGFR3) in the retinas of Kimba and Akimba mice by RT-PCR ($P < 0.005$). Furthermore, lymphangiogenesis was not present in aged Kimba or Akimba mice. Significantly increased numbers of Lyve1-positive cells present in the retinas of Kimba and Akimba mice, especially in the peripheral areas, were CD11b positive, indicating a macrophage population ($P < 0.005$). VEGF-C in PDR vitreous with vitreous hemorrhage (VH) was higher than in PDR without VH or a macular hole.

CONCLUSIONS. Retinal VEGF-A overexpression did not cause typical lymphangiogenesis despite upregulated lymphatic-related factors and significant Lyve1-positive macrophage infiltration.

Keywords: lymphatic, angiogenesis, diabetic retinopathy, vitrectomy, transgenic mice, aging, podoplanin, Prox1

The lymphatic system is important for the removal of interstitial fluid and macromolecules, including proteins, their transport to lymph nodes before entering the blood circulation, and the transport of immune cells to the lymph nodes, as part of the immune system.[1] The discovery of lymphatic endothelial markers including podoplanin, lymphatic endothelial hyaluronan receptor-1 (Lyve-1), and prospero homeobox 1 (Prox1) has promoted lymphatic research in physiological and pathological conditions.[2–4] It is thought that lymphatics are present in most organs, with the exception of the central nervous system, bone marrow, and avascular tissues including the cartilage, cornea, and epidermis.[1] However, a recent study reported that the brain contains functional lymphatic vessels that line the dural sinuses.[5] The retina and brain develop as part of the central nervous system.[6] Although the eye contains lymphatics in the conjunctiva and cornea limbus,[7,8] it is generally accepted that there is no lymphatic system in the mammalian retina.[1,7]

Lymphangiogenesis usually occurs in the fetal period, as well as angiogenesis, which is separated from the blood vessels of the venous system.[9] Pathological conditions such as cancer and inflammation induce angiogenesis and lymphangiogenesis.[1] The current paradigm regards lymphangiogenesis secondary to angiogenesis.[10,11] Vascular endothelial growth factor (VEGF)-A and -C are involved in angiogenesis and lymphangiogenesis in various diseases.[12–14] We previously reported that lymphangiogenesis was not observed in a mouse model of choroidal neovascularization.[15] However, whether lymphangiogenesis occurs in retinas with a high expression of VEGF-A has not been reported.

Diabetic retinopathy (DR) is a growing cause of visual impairment worldwide and remains a serious problem.[16]

1

Continuous basic research and clinical studies have shown that overexpressed VEGF-A contributes to interstitial fluid retention in diabetic macular edema (DME) and retinal angiogenesis in proliferative diabetic retinopathy (PDR), which can cause visual impairment.[17] A previous study of single nucleotide polymorphisms (SNPs) reported genetic variation in the *VEGFC* gene was associated with the presence of DR and DME, suggesting the contribution of VEGF-C to the pathogenesis of DR.[18] Several clinical studies using vitreous samples have reported that VEGF-C is not elevated in DR.[19,20] Another recent study showed PDR neovascular tissues developed Prox1-positive capillary sprouts with lymphatic endothelial structures ex vivo,[21] suggesting the potential ability of the retina to induce lymphangiogenesis. However, whether lymphangiogenesis occurs in DR is unclear.

METHODS

Animals

All animal experiments were approved by the Animal Care Committee of Kyushu University (A19-01401). All experimental procedures on the animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. As a model of type 1 diabetes, heterozygous Akita (Ins2^{Akita}) mice on a C57BL/6 background and C57BL/6JJcl mice were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan).[22] Kimba mice (trVEGF029), a model of hVEGF-induced retinal neovascularization[23] were kindly provided by Professor Elizabeth Rakoczy (University of Western Australia). All mice had access to water and standard laboratory chow as desired and were housed in an air-conditioned room with a 12-hour light and dark cycle according to institutional guidelines. Seven-to eight-week-old mice were considered "young," and six-and 12-month-old mice were considered "aged" were used for experiments.

Generation of the Akimba Mouse Model

Heterozygous Kimba (*vegfa*$^{+/-}$) mice were generated as described previously.[23] Homozygous Kimba (*vegfa*$^{+/+}$) mice were mated with mice heterozygous for the Akita spontaneous mutation (Ins2^{Akita}), to generate Kimba or Akimba (Ins2^{Akita} *vegfa*$^{+/-}$) mice.[24]

Generation of Lyve1 Positive Kimba Mice

Homozygous Kimba (*vegfa*$^{+/+}$) mice were mated with Lyve1-positive mice (*Lyve1*$^{EGFP/Cre}$) (012601; Jackson Laboratory, Bar Harbor, ME, USA), to generate Lyve1-positive Kimba (*Lyve1*$^{EGFP/Cre}$ *vegfa*$^{+/-}$) mice.

Genotyping

DNA was isolated from tail clips of mice. Akita mice were genotyped for the *Ins2* gene as described (Jackson Laboratory). Genotyping of Kimba mice was carried out as previously described.[23] Lyve1-positive mice were genotyped for the *Lyve1* gene as described (Jackson Laboratory). Akimba mice were genotyped by using protocols for Kimba and Akita mice.[24] Lyve1-positive Kimba mice were genotyped by using protocols for Kimba and Lyve1-positive mice.

Fluorescein Angiography

Mice were anesthetized, their pupils dilated, and 0.1 ml 2.5% fluorescein sodium (Novartis, Basel, Switzerland) was injected intraperitoneally. Vascular leakage was evaluated using the Optos California ultrawide-field imaging system (Optos Inc., Marlborough, MA, USA) at the late phase (two to three minutes). Color fundus images were also taken as previously described.[25]

Immunofluorescence

Mouse eyes were enucleated and fixed with 4% paraformaldehyde for 30 minutes at 4°C. For whole-mount preparation, the retinas were microsurgically exposed by removing other portions of the eye. Tissues were placed in methanol for 20 minutes and washed with Tween PBS (TPBS) once for 15 minutes. Tissues were incubated overnight at 4°C with anti-mouse CD31 mAb (550274; BD PharMingen, San Diego, CA, USA; 1:100), anti-mouse Lyve1 Ab (11-034; AngioBio, Del Mar, CA, USA; 1:100), anti-hamster podoplanin Ab (11-033, AngioBio; 1:200), FITC anti-mouse CD80 (B7-1) (11-0801-81; eBioscience, San Diego, CA, USA; 1:40) , FITC anti-mouse CD206 (141703; BioLegend, San Diego, CA, USA; 1:100), and Alexa Fluor 647 anti-mouse F4/80 (MCA497A647; AbD Serotec, Kidlington, UK; 1:200) diluted in PBS containing 10% goat serum and 1% Triton X-100. Tissues were washed four times for 15 minutes each in TPBS followed by incubation with Alexa Fluor 488 goat anti-rat IgG (A-11006; Invitrogen/Thermo Fisher Scientific, Inc., Kalamazoo, MI, USA; 1:200), Alexa Fluor 546 goat anti-rabbit IgG (A-11035; Invitrogen/Thermo Fisher Scientific, Inc.; 1:200), and Alexa Fluor 647 goat anti-hamster IgG (ab173004; Abcam/Thermo Fisher Scientific, Inc.; 1:200) overnight at 4°C. The flat mounts were prepared on glass slides using mounting medium (TA-030-FM Mountant Permafluor; Lab Vision Corporation/Thermo Fisher Scientific, Inc.). The eyecups were embedded in OCT compound (Tissue-Tek, Sakura, Torrance, CA, USA) and were vertically sectioned at 10-μm thickness using a Leica CM1900 cryostat (Leica, Wetzlar, Germany). The flat mounts were examined by fluorescence microscopy (Leica TCS SP2 laser scanning confocal microscope; Leica Microsystems GmbH).

Real-Time PCR Analyses

Total RNA was extracted from whole retinas at selected time points. The MagDEA RNA kit (Precision System Science, Pleasanton, CA, USA) was used according to the protocol included with the kit. RNA concentrations were quantified and cDNA was synthesized with a First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Quantitative RT-PCR was performed and analyzed using TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) and a LightCycler 96 Real-time PCR System (Roche). Twenty microliters of the PCR mixture containing 1 μL of primer were loaded into each LightCycler well. An initial step of 10 minutes at 95°C was used to activate the HotStart DNA polymerase, and then a two-step cycling program including 45 cycles of 95°C for 20 seconds and 60°C for 40 seconds was used for the TaqMan assays. The LightCycler 96 Real-time PCR System software was used to detect the probe, calculate the threshold cycles (Ct values), and perform additional analyses. Gene expression was normalized to *Gapdh* mRNA

levels. The sequences of gene-specific primers are shown in Supplementary Table S1.

Human Vitreous Fluid Collection and Measurements of VEGF Family Members

This study was conducted according to the principles of the Declaration of Helsinki. After approval from the Institutional Review Board of Kyushu University Hospital (UMIN000014724), informed consent regarding the use of vitreous fluid was obtained from each patient with PDR or a macular hole (MH). PDR patients had not been treated with anti-VEGF drugs to avoid a decrease in VEGF and had not undergone retinal photocoagulation for at least six months. A vitreous sample was obtained while performing pars plana vitrectomy surgery. The sample was taken in sterile conditions, using a plastic tube of 1.5 mL volume. Then, 0.5 to 1.0 mL of undiluted vitreous was aspirated before each opening of the infusion line. The sample of vitreous was centrifuged and stored in a refrigerator at $-80°C$. Levels of cytokines in the vitreous were determined by ELISA. The quantitative values of VEGF-A, -C, -D, and soluble VEGF receptor 2 concentrations in the vitreous were measured with Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA; code numbers: DVE00, DVEC00, DVED00, and DY357, respectively)

RESULTS

Fundus Imaging in Akita, Kimba, and Akimba Mice

Fundus photography and fluorescein angiography (FA) showed no retinal angiogenesis in Akita mice or wild-type (WT) mice, whereas retinal hemorrhage, white spots representing exudative changes, and abnormal retinal blood vessels by fundus photography, and retinal neovascularization with increased permeability by FA were observed in Kimba and Akimba mice (Fig. 1A).

Lymphatic-Related Factors in the Retinas of Akita, Kimba, and Akimba Mice

RT-PCR was performed to investigate the expressions of lymphatic-related factors (*Lyve1, Podoplanin, Prox1, VEGF-A, VEGF-C, VEGF-D, VEGFR2, VEGFR3, and PECAM-1*) in the retinas of WT, Akita, Kimba, and Akimba mice. All genes except for *Prox1* were upregulated in Kimba and Akimba mice compared with WT mice, whereas *Prox1* was significantly upregulated in Akimba mice only. Akita mice showed a significant upregulation of *Lyve1* and *VEGF-A* compared with WT mice (Fig. 1B).

Retinal Lyve1 Expression in Akita, Kimba, and Akimba Mice

To investigate the presence of lymphangiogenesis in retinas of Akita, Kimba, and Akimba mice, Lyve1 immunostaining was performed using the whole mount. The conjunctiva and cornea from WT mice were used as positive controls. We confirmed lymphatic vessels were stained for CD31, Lyve1, and podoplanin as previously reported[10] (Supplementary Fig. S1). These lymphatic vessels could be distinguished from vascular vessels (Lyve1⁻CD31⁺Podoplanin⁻)

(Supplementary Fig. S1). Furthermore, Lyve1-positive cells observed in the conjunctiva were previously reported to be macrophages[26,27] (Supplementary Fig. S1). Next, we used the same antibodies to examine 110 mouse retinas (23 retinas = WT, 15 retinas = Akita, 46 retinas = Kimba, 26 retinas = Akimba). CD31⁺ vasculature, but not Lyve1-positive luminal structures, was observed in all retinas except for the retina of one Kimba mouse (Fig. 2A and Supplementary Fig. S2). However, Lyve1-positive dendriform cells were observed in all mouse models (Fig. 2A). Quantitative analysis showed the number of Lyve1-positive cells was significantly increased in Kimba and Akimba mice compared with WT and Akita mice (Fig. 2B). Furthermore, there was no difference in the number of Lyve1-positive cells in WT and Akita mice regardless of the localization, whereas Kimba and Akimba mice had significantly more Lyve1-positive cells in the middle or periphery compared with the center of the retina (Figs. 2C, 2D, 2E). These results suggested that Lyve1-positive cells are increased in mouse retinas (especially in the periphery) with VEGF overexpression but not with diabetic status.

Lyve1-Positive Cells in the Retina Express CD11b

To investigate the characteristics of Lyve1-positive cells, Lyve1-GFP mice were crossed with Kimba mice to generate Lyve1-GFP–expressing Kimba mice. A previous study by Xu et al.[27] showed Lyve1-positive cells were macrophages in normal C57BL/6 murine retinas. Immunostaining of retinas from Kimba mice showed that all Lyve1-positive cells expressed CD11b, indicating the increased Lyve1-postive cells in VEGF-overexpressing retina are a macrophage population (Fig. 3).

Phenotype of Lyve1-Positive Macrophages in the Retinas of Akita and Kimba Mice

To investigate whether phenotypic differences in Lyve1-positive macrophages affected the retinal pathology between Akita and Kimba mice, we examined macrophage markers. Immunohistochemistry with antibodies to CD80 and CD206 (representative markers of M1- and M2-like macrophages, respectively[28]) showed Lyve1-positive macrophages expressed CD206 but not CD80 in both models, although CD80⁺ F4/80⁺ macrophages were present in the retinas as a positive control (Supplementary Fig. S3A and S3B). These data suggest that Lyve1-positive macrophages have an M2-like phenotype in both models.

Long-Term Observation of VEGF Overexpression in Mice

Lymphangiogenesis occurs after angiogenesis in certain conditions such as VEGF-A-overexpressing cornea.[11] To investigate whether lymphangiogenesis was induced in the late phase of angiogenesis, we used aged Kimba mice (n = 11, six 6-month-old retinas and five 12-month-old retinas) with the potential long-term presence of retinal angiogenesis. First, fundus photographs and FA images confirmed the presence of neovascularization in all 6-month-old Kimba mice (Fig. 4A). Interestingly, two retinas (40%) from 12-month-old Kimba mice showed entire retinal vessel occlusion, whereas three retinas showed apparent angiogenesis (Fig. 4A). Next, RT-PCR was performed to investigate lymphatic-related factors using the retinas of six- and
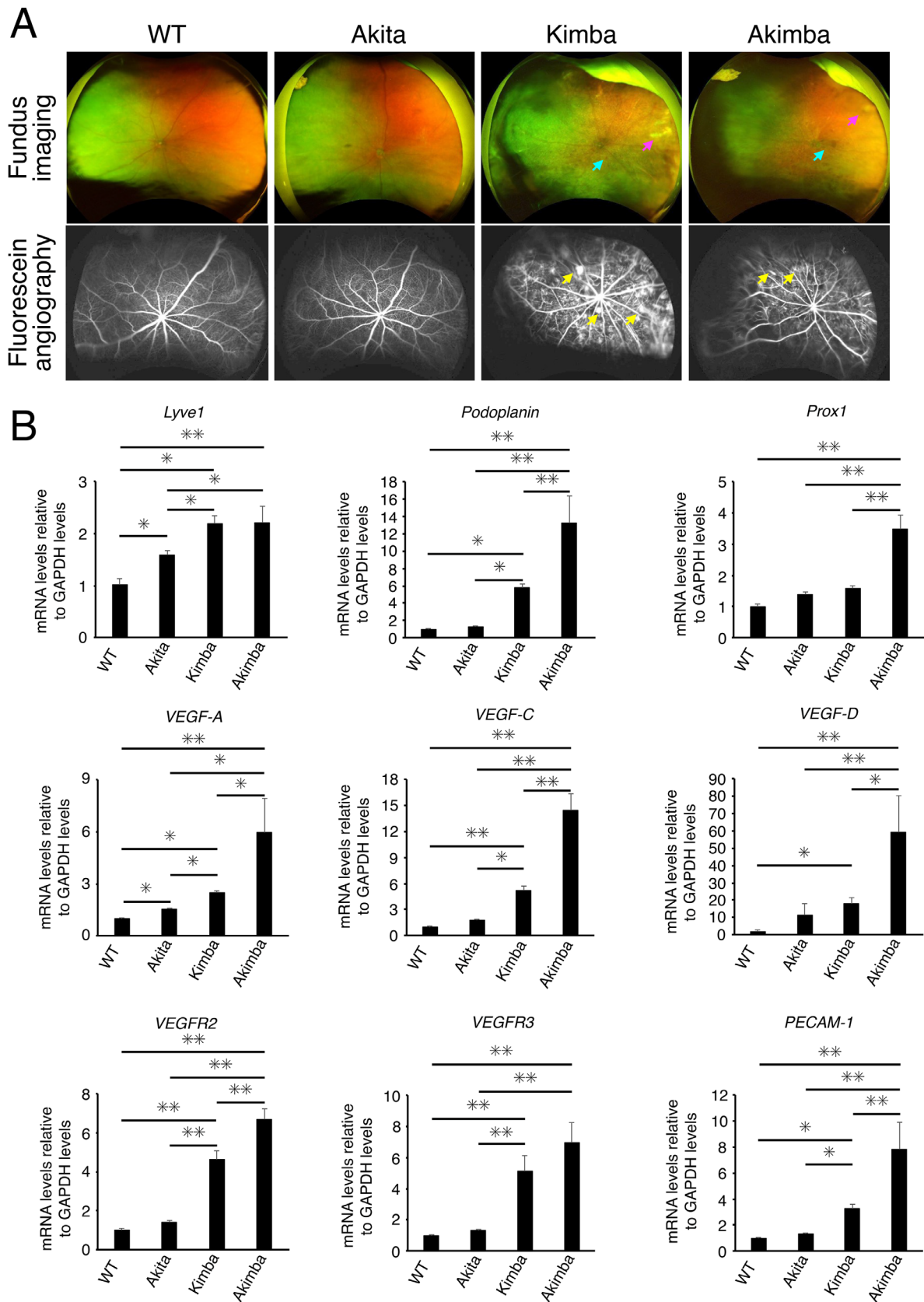
FIGURE 1. Characteristics of mouse models of diabetic retinopathy. **(A)** Representative fundus images and fluorescein angiography (late phase; 2–3 minutes after dye injection) of C57BL/6 WT mice and three different mouse models of diabetic retinopathy (DR) (seven to eight weeks old). These were imaged using an Optos California ultrawide-field imaging system. The three mouse models were heterozygous Akita (Ins2^{Akita}) as a model of type 1 diabetes, and Kimba (*vegfa$^{+/+}$*), and Akimba (Ins2^{Akita} *vegfa$^{+/-}$*) mice. *Blue, pink*, and *yellow arrows*

indicate retinal hemorrhage, exudates, and retinal angiogenesis, respectively. **(B)** Expressions of the mRNAs of various cytokines related to lymphogenesis and angiogenesis in WT, Akita, Kimba, and Kimba retinas (n = 20). Values are the means ± SD. $^*P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.
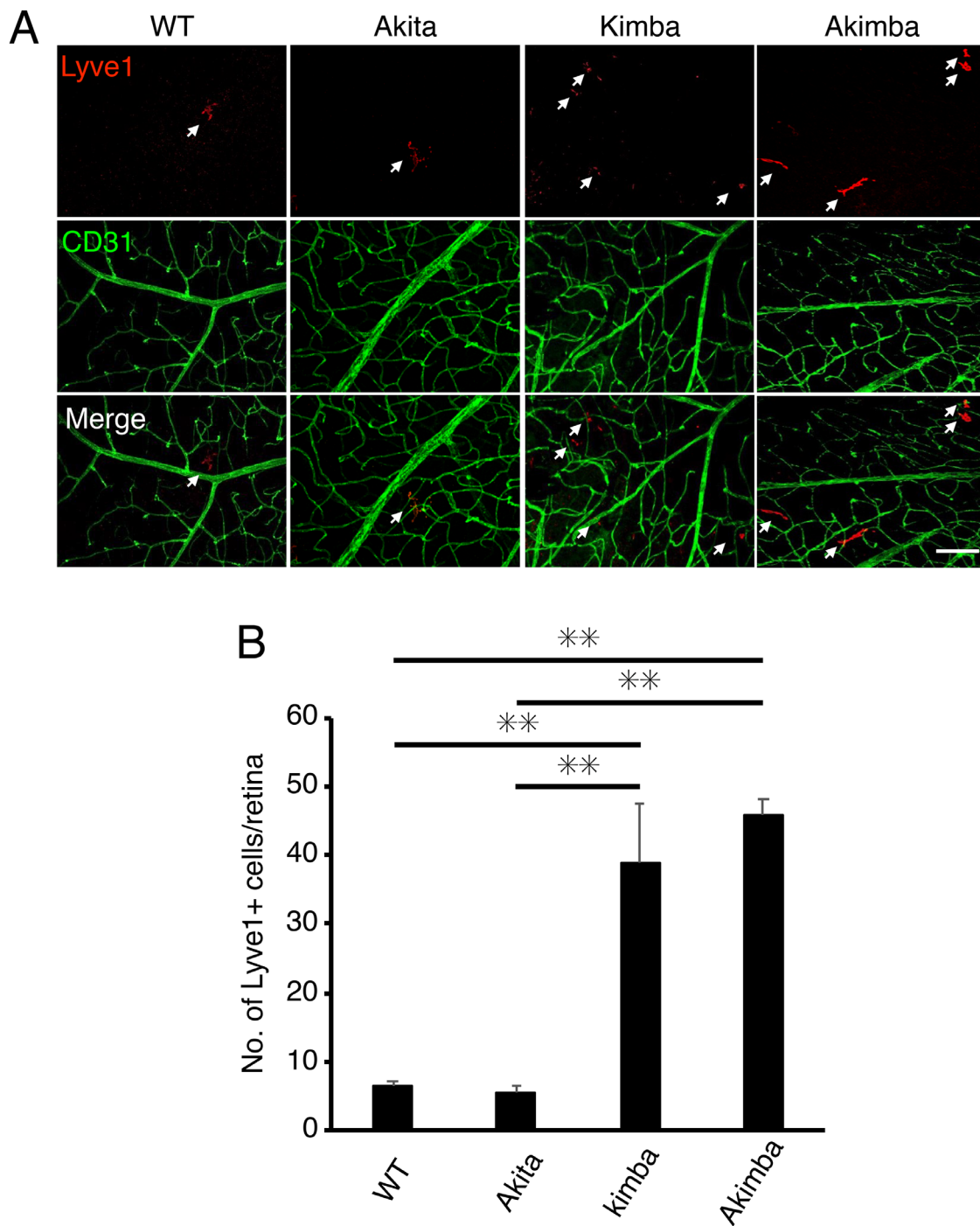

FIGURE 2. Lyve1 expression in mouse models of diabetic retinopathy. **(A)** Representative images of flat-mounted retinas from WT, Akita (Ins2^{Akita}), Kimba (*vegfa*$^{+/+}$), and Akimba (Ins2^{Akita} *vegfa*$^{+/-}$) mice (7- to 8-week-old) with staining for Lyve1 (*red*) and CD31 (*green*). *White arrows* indicate intraretinal Lyve1-positive cells. *Scale bar*: 50 µm. **(B)** Quantitation of the number of Lyve1-positive cells per whole retina from WT, Akita, Kimba, and Akimba mice (n = 8). Values are the means ± SD. $^{**}P < 0.01$, Student's *t*-test. **(C)** A representative image of flat-mounted retinas from Kimba mice with staining for CD31 (*green*) shows areas that were quantified in Figure 2D (*white squares*). **(D)** Representative images in the center, middle, and periphery of flat-mounted retinas from WT, Akita, Kimba, and Akimba mice with staining for CD31 (*green*) and F4/80 (*red*). *Scale bar*: 50 µm. **(E)** Quantitation of the number of Lyve1-positive cells per indicated area of WT, Akita, Kimba, and Akimba mice (n = 12). Values are the means ± SD. $^*P < 0.05$, $^{**}P < 0.005$, Student's *t*-test.
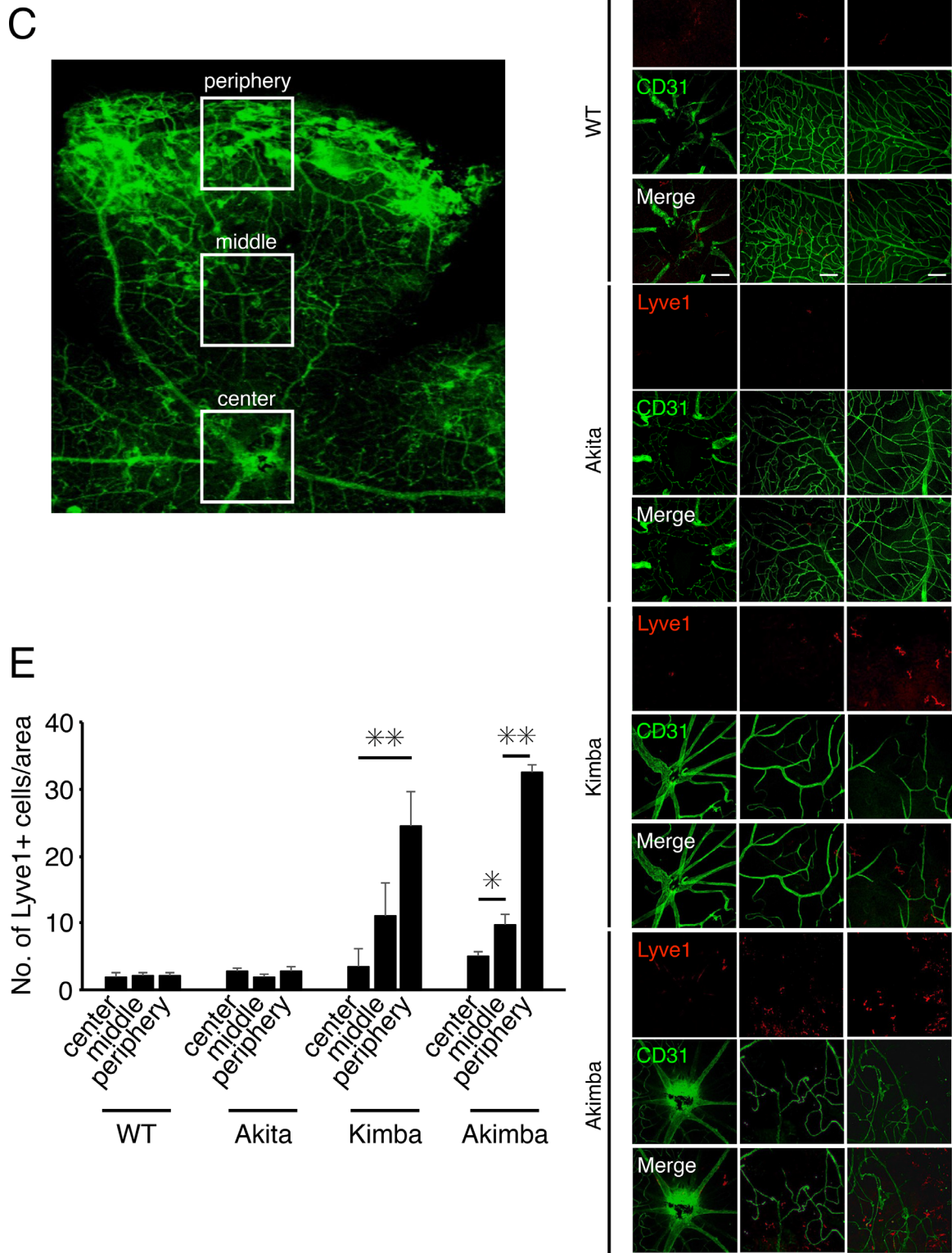
C

D

E

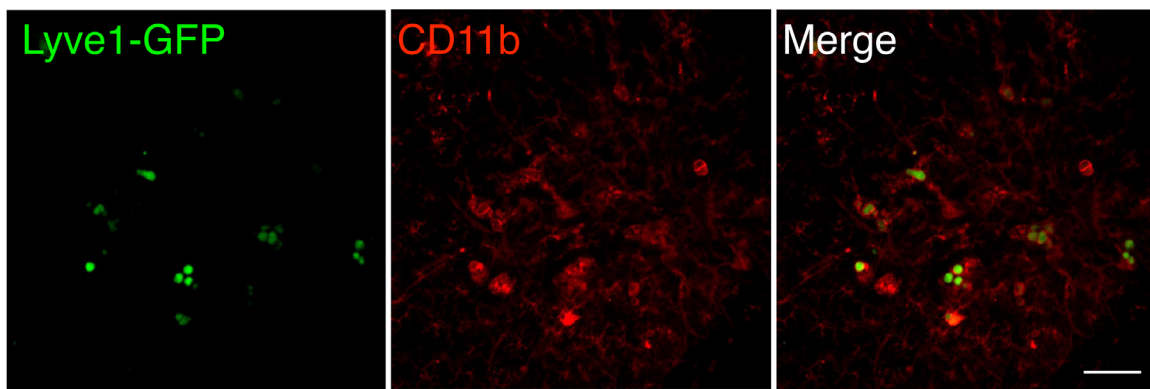

Figure 2. Continued

Lyve1-GFP x Kimba mouse

Figure 3. Population of Lyve1-positive Cells in Kimba Mice. Representative images of flat-mounted retinas of Lyve1-GFP (*green*) × Kimba mice (*vegfa*$^{+/+}$) (*Lyve1*$^{EGFP/Cre}$ *vegfa*$^{+/-}$, 7- to 8-week-old) with staining for CD11b (*red*). The image shows CD11b cells with GFP expressed in the nucleus. *Scale bar*: 50 μm.

12-month-old Kimba mice. All lymphatic-related factors in the retina were significantly decreased in aged mice compared with young Kimba mice (Fig. 4B). Furthermore, immunostaining showed that the retinas of aged Kimba mice did not show obvious lymphangiogenesis. Lyve1-positive cells accumulated in perivascular areas that did not express podoplanin (Fig. 4C and Supplementary Fig. S4).

Vitreous Lymphatic-Related Factors in Diabetic Retinopathy Patients

Finally, the levels of lymphatic-related factors (VEGF-A, -C, -D, and sVEGFR2) in vitreous fluid from PDR patients were examined by ELISA. VEGF-A was significantly higher in vitreous fluid from PDR patients with and without vitreous hemorrhage compared with those with macular holes (Fig. 5A). Interestingly, the level of VEGF-C in the vitreous fluid was similar to that of MH in PDR without vitreous hemorrhage, but significantly higher in PDR with vitreous hemorrhage compared with other patients (Fig. 5B). The levels of VEGF-D and sVEGFR2 in the vitreous fluid were similar in PDR and MH patients (Figs. 5C and 5D).

Discussion

Lymphatic vessels are widely accepted to be absent in the retina and have not been identified.[7] In general, lymphangiogenesis was observed to occur in association with angiogenesis under various pathological conditions.[1] In this study, we investigated whether lymphangiogenesis occurred in DR with retinal angiogenesis. We investigated three animal models of DR and did not detect lymphangiogenesis except in one eye. However, we found the increased expression of the lymphangiogenic factors VEGF-A, VEGF-C, and VEGF-D, as well as the increased expression of the lymphangiogenic markers Lyve1, podoplanin, and VEGFR3. We also found increased Lyve1-positive cells in Kimba and Akimba mice, which represent a macrophage population and a potential precursor of lymphangiogenesis, as previously reported.[27,29] To investigate further the possibility of lymphangiogenesis in the late stage of VEGF-A expression, the same study was conducted in six- and 12-month-old mice, but no lymphangiogenesis was observed. In the vitreous of patients with PDR with vitreous hemorrhage, we observed a significant increase in VEGF-C expression. This study suggests that, unlike in cancer and the cornea, increased VEGF-A expression is not sufficient to induce lymphangiogenesis regardless of increased VEGF-C expression and Lyve1-positive macrophage infiltration.

VEGF-A is a known inducer of angiogenesis as well as lymphangiogenesis, and Cursiefen et al.[13] previously reported that VEGF-A induced lymphangiogenesis in the cornea by upregulating VEGF-C and -D expressions via macrophage recruitment. Recently, we reported that macrophage fractional infiltration occurred in Kimba and Akimba mice.[30] In this study, we also found that VEGF-C and -D were significantly elevated in the retinas of these mice. However, lymphangiogenesis was not observed in most mouse retinas, which is consistent with a previous report of VEGF-C expression in uveal melanoma.[31] This may be because of the lack of pre-existing lymphatic vessels in the retina. In addition, unlike other tissues such as the cornea, factors that inhibit lymphangiogenesis, such as thrombospondin-1 may be involved.[32]

Previously, Maruyama et al.[29] showed that lymphatic vessels were formed by Lyve1-positive CD11b-positive macrophages. In the present study, we also observed an increase in Lyve1-positive macrophages, which were CD11b positive. However, no tube-like structures with Lyve1-positive cells were observed in the retina. Furthermore, it was reported that Lyve1-positive cells are a fraction of perivascular cells.[33] Perivascular Lyve1-positive macrophages prevent arterial stiffness by controlling the expression of collagen in vascular smooth muscle cells, a process dependent on the engagement of Lyve1 with hyaluronan on smooth muscle cells. In the present study, perivascular Lyve1-positive cells were also observed, especially in aged mice with VEGF-A overexpression. Although Lyve1-positive cells might maintain retinal homeostasis instead of lymphatic vessels, it remains to be seen what role they play in retinas with high VEGF-A levels. The choroid is also reported to contain abundant Lyve1-positive macrophages without the presence of classic lymphatics.[34]
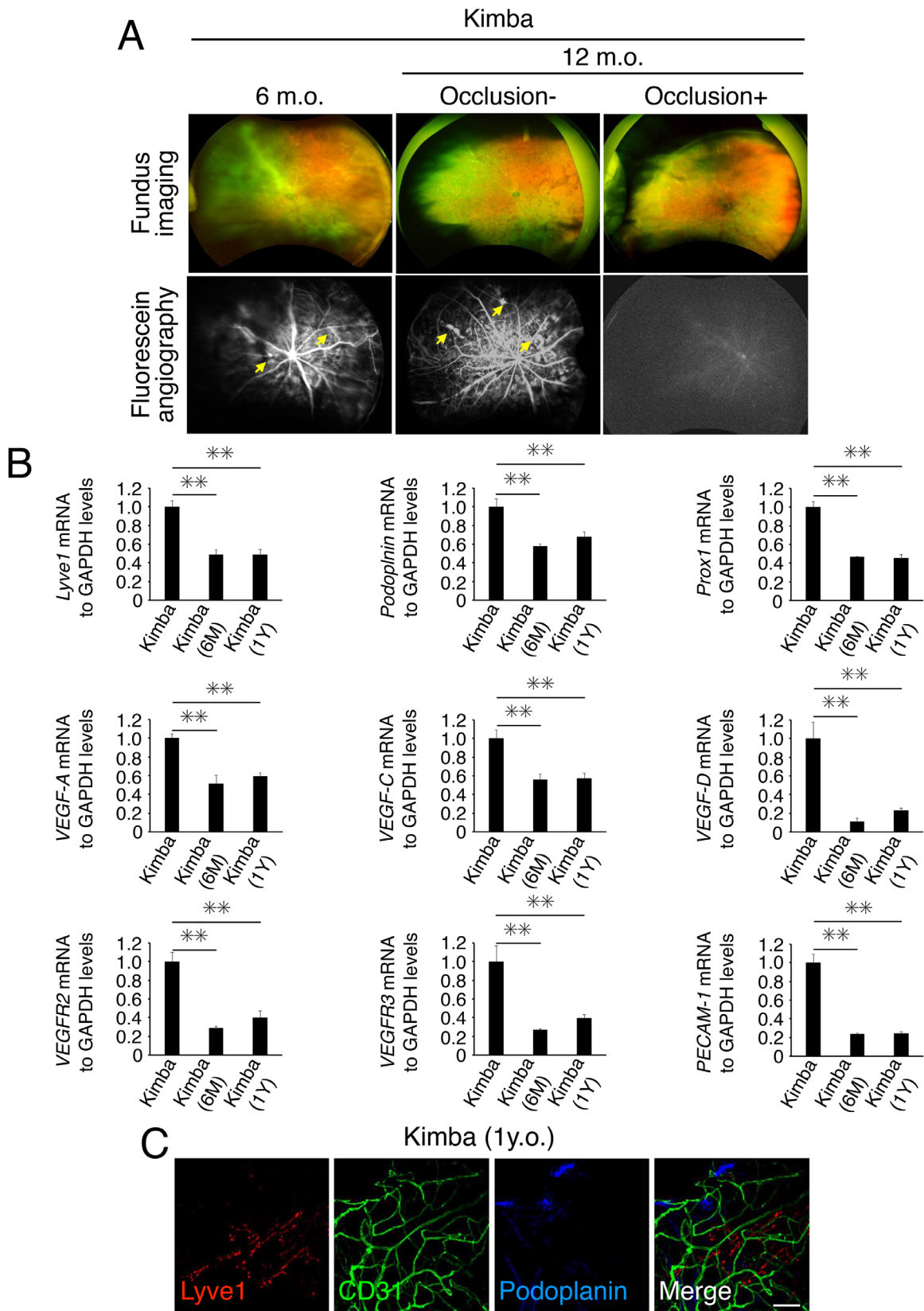
FIGURE 4. Characteristics of aged Kimba mice. **(A)** Representative fundus images and fluorescein angiography (late phase; two to three minutes after dye injection) of C57BL/6 WT mice and three different mouse models of diabetic retinopathy (DR) (6- or 12-month-old). These were imaged using an Optos California ultrawide-field imaging system. *Yellow arrows* indicate retinal angiogenesis. **(B)** Expressions of the mRNAs of various cytokines related to lymphogenesis and angiogenesis in retinas from seven- to eight-week-old, six-month-old, and 12-month-old Kimba mice (n = 6). Values are the means ± SD. [**]*P* < 0.01, Student's *t*-test. **(C)** Representative images of flat-mounted retinas from 12-month-old Kimba mice with staining for Lyve1 (*red*), CD31 (*green*), and podoplanin (*blue*). *Scale bar*: 50 μm.
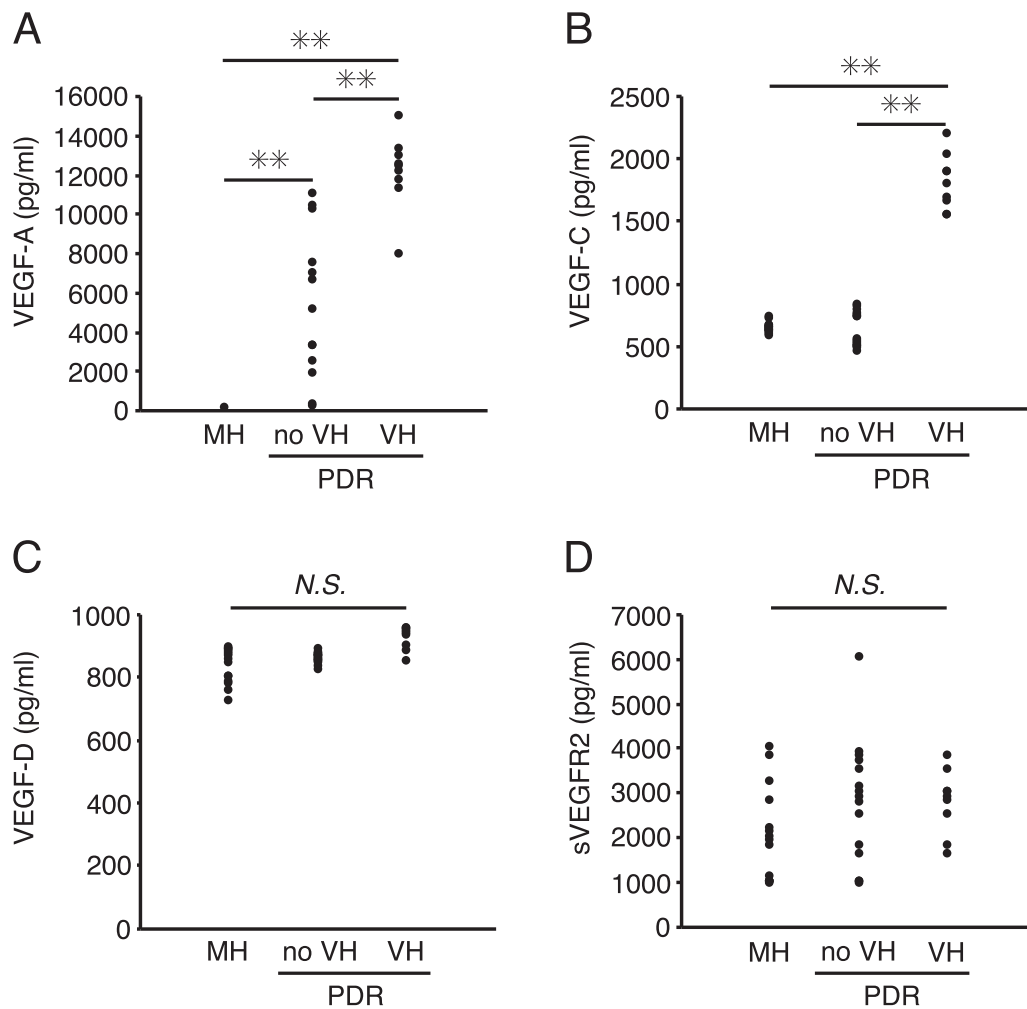
FIGURE 5. Measurement of lymphatic-related factors in the vitreous fluid of proliferative diabetic retinopathy with and without vitreous hemorrhage. VEGF-A **(A)**, VEGF-C **(B)**, VEGF-D **(C)**, and VEGFR2 expression in vitreous fluids from patients with a macular hole (MH) (n = 15), proliferative diabetic retinopathy (PDR) without vitreous hemorrhage (VH) (n = 15), and PDR with VH (n = 9). All cytokines were measured by ELISA. Values are the means ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$, Student's *t*-test.

These Lyve1-positive macrophages in the posterior segment may be involved in the metabolism of hyaluronan, a major constituent of the vitreous.[35]

In this study, angiogenesis was observed in Kimba, but not Akita, mice. Although we previously observed the involvement of M2-like macrophages in retinal angiogenesis,[36] Lyve1-positive macrophages expressed M2-like markers in both mouse models. The difference in retinal pathology between the two models may depend on the number of Lyve1-positive macrophages rather than their phenotype. The origin of Lyve1-positive macrophages and their involvement in pathogenesis require further investigation.

Surprisingly, we observed lymphatic vessel invasion into the retina in only one eye of a Kimba mouse (Supplementary Fig. S2). However, this phenomenon was not observed in any other mice. This eye might have suffered trauma or have been infected because lymphatic vessels can invade into the eye in cases of open globe injuries caused by trauma.[37] Therefore lymphatic vessels might invade the retina under certain conditions such as potent inflammation.

In this study, VEGF-A was significantly upregulated in PDR compared with MH, whereas VEGF-C and VEGF-D expressions were similar between PDR and MH. Previous studies reported that VEGF-C was not upregulated in the vitreous of PDR and that VEGF-C levels did not correlate with PDR progression.[19,20] However, we found that when PDR cases were differentiated by the presence or absence of vitreous hemorrhage, VEGF-C expression was significantly increased in the vitreous hemorrhage group compared with those without vitreous hemorrhage. This suggests that VEGF-C is elevated in PDR with higher disease activity or that VEGF-C in the blood accumulates in the vitreous. An ex vivo study with fibrovascular membranes from patients with PDR reported that lymphovascular-like structures in conjunction with inflammation were present under conditions containing VEGF-A and VEGF-C.[21] This suggests that a complex ischemic and inflammatory microenvironment, rather than a single factor, may promote the formation of lymph-like vessels.

Recently, the presence of lymphatic vessels was reported in normal mouse and human brain,[5] although retinal lymphatic vessels were not identified in this study using animals. In DR, the vascular permeability is increased.[38] Generally, these exudates are absorbed by the pump

function of retinal pigment epithelial cells, and it is thought that a decrease in this pump function might contribute to macular edema in DR.[39] However, the lack of lymphatic vessels in the retina may also be a cause of chronic DME. Although anti-VEGF therapy is the first choice for DME treatment,[40,41] recent clinical data reported that approximately 40% of DME is resistant to anti-VEGF therapy.[42] In the future, the development of therapies that enhance the lymphatic function of the retina may be useful in these types of cases.

Acknowledgments

References

1. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature*. 2005;438:946–953.

2. Banerji S, Ni J, Wang SX, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol*. 1999;144:789–801.

3. Breiteneder-Geleff S, Soleiman A, Kowalski H, et al. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol*. 1999;154:385–394.

4. Wigle JT, Harvey N, Detmar M, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J*. 2002;21:1505–1513.

5. Louveau A, Smirnov I, Keyes TJ, et al. Structural and functional features of central nervous system lymphatic vessels. *Nature*. 2015;523:337–341.

6. Harada T, Harada C, Parada LF. Molecular regulation of visual system development: more than meets the eye. *Genes Dev*. 2007;21:367–378.

7. Nakao S, Hafezi-Moghadam A, Ishibashi T. Lymphatics and lymphangiogenesis in the eye. *J Ophthalmol*. 2012;2012:783163.

8. Bock F, Maruyama K, Regenfuss B, et al. Novel anti(lymph)angiogenic treatment strategies for corneal and ocular surface diseases. *Prog Retin Eye Res*. 2013;34:89–124.

9. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell*. 1999;98:769–778.

10. Nakao S, Maruyama K, Zandi S, et al. Lymphangiogenesis and angiogenesis: concurrence and/or dependence? Studies in inbred mouse strains. *FASEB J*. 2010;24:504–513.

11. Nakao S, Zandi S, Hata Y, et al. Blood vessel endothelial VEGFR-2 delays lymphangiogenesis: an endogenous trapping mechanism links lymph- and angiogenesis. *Blood*. 2011;117:1081–1090.

12. Nagy JA, Vasile E, Feng D, et al. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med*. 2002;196:1497–1506.

13. Cursiefen C, Chen L, Borges LP, et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest*. 2004;113:1040–1050.

14. Cao R, Eriksson A, Kubo H, Alitalo K, Cao Y, Thyberg J. Comparative evaluation of FGF-2-, VEGF-A-, and VEGF-C-induced angiogenesis, lymphangiogenesis, vascular fenestrations, and permeability. *Circ Res*. 2004;94:664–670.

15. Nakao S, Zandi S, Kohno R, et al. Lack of lymphatics and lymph node-mediated immunity in choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2013;54:3830–3836.

16. Leasher JL, Bourne RR, Flaxman SR, et al. Global Estimates on the Number of People Blind or Visually Impaired by Diabetic Retinopathy: A Meta-analysis From 1990 to 2010. *Diabetes Care*. 2016;39:1643–1649.

17. Duh EJ, Sun JK, Stitt AW. Diabetic retinopathy: current understanding, mechanisms, and treatment strategies. *JCI Insight*. 2017;2:e93751.

18. Kaidonis G, Burdon KP, Gillies MC, et al. Common Sequence Variation in the VEGFC Gene Is Associated with Diabetic Retinopathy and Diabetic Macular Edema. *Ophthalmology*. 2015;122:1828–1836.

19. Kovacs K, Marra KV, Yu G, et al. Angiogenic and Inflammatory Vitreous Biomarkers Associated With Increasing Levels of Retinal Ischemia. *Invest Ophthalmol Vis Sci*. 2015;56:6523–6530.

20. Wu F, Phone A, Lamy R, et al. Correlation of Aqueous, Vitreous, and Plasma Cytokine Levels in Patients With Proliferative Diabetic Retinopathy. *Invest Ophthalmol Vis Sci*. 2020;61:26.

21. Gucciardo E, Loukovaara S, Korhonen A, et al. The microenvironment of proliferative diabetic retinopathy supports lymphatic neovascularization. *J Pathol*. 2018;245:172–185.

22. Barber AJ, Antonetti DA, Kern TS, et al. The Ins2Akita mouse as a model of early retinal complications in diabetes. *Invest Ophthalmol Vis Sci*. 2005;46:2210–2218.

23. Lai CM, Dunlop SA, May LA, et al. Generation of transgenic mice with mild and severe retinal neovascularisation. *Br J Ophthalmol*. 2005;89:911–916.

24. Rakoczy EP, Ali Rahman IS, Binz N, et al. Characterization of a mouse model of hyperglycemia and retinal neovascularization. *Am J Pathol*. 2010;177:2659–2670.

25. Nakao S, Arita R, Nakama T, et al. Wide-field laser ophthalmoscopy for mice: a novel evaluation system for retinal/choroidal angiogenesis in mice. *Invest Ophthalmol Vis Sci*. 2013;54:5288–5293.

26. Chen L, Cursiefen C, Barabino S, Zhang Q, Dana MR. Novel expression and characterization of lymphatic vessel endothelial hyaluronate receptor 1 (LYVE-1) by conjunctival cells. *Invest Ophthalmol Vis Sci*. 2005;46:4536–4540.

27. Xu H, Chen M, Reid DM, Forrester JV. LYVE-1-positive macrophages are present in normal murine eyes. *Invest Ophthalmol Vis Sci*. 2007;48:2162–2171.

28. Zandi S, Nakao S, Chun KH, et al. ROCK-isoform-specific polarization of macrophages associated with age-related macular degeneration. *Cell Rep*. 2015;10:1173–1186.

29. Maruyama K, Ii M, Cursiefen C, et al. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest*. 2005;115:2363–2372.

30. Arima M, Nakao S, Yamaguchi M, et al. Claudin-5 Redistribution Induced by Inflammation Leads to Anti-VEGF-Resistant Diabetic Macular Edema. *Diabetes*. 2020;69:981–999.

31. Clarijs R, Schalkwijk L, Ruiter DJ, de Waal RM. Lack of lymphangiogenesis despite coexpression of VEGF-C and its receptor Flt-4 in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:1422–1428.

32. Cursiefen C, Maruyama K, Bock F, et al. Thrombospondin 1 inhibits inflammatory lymphangiogenesis by CD36 ligation on monocytes. *J Exp Med*. 2011;208:1083–1092.

33. Lim HY, Lim SY, Tan CK, et al. Hyaluronan Receptor LYVE-1-Expressing Macrophages Maintain Arterial Tone through Hyaluronan-Mediated Regulation of Smooth Muscle Cell Collagen. *Immunity*. 2018;49:1191.

34. Schroedl F, Brehmer A, Neuhuber WL, Kruse FE, May CA, Cursiefen C. The normal human choroid is endowed with a significant number of lymphatic vessel endothelial hyaluronate receptor 1 (LYVE-1)-positive macrophages. *Invest Ophthalmol Vis Sci*. 2008;49:5222–5229.

35. Le Goff MM, Bishop PN. Adult vitreous structure and postnatal changes. *Eye (Lond)*. 2008;22:1214–1222.

36. Zhou Y, Yoshida S, Nakao S, et al. M2 Macrophages Enhance Pathological Neovascularization in the Mouse Model of Oxygen-Induced Retinopathy. *Invest Ophthalmol Vis Sci*. 2015;56:4767–4777.

37. Wessel JM, Hofmann-Rummelt C, Kruse FE, Cursiefen C, Heindl LM. Invasion of lymphatic vessels into the eye after open globe injuries. *Invest Ophthalmol Vis Sci*. 2012;53:3717–3725.

38. Murata T, Nakagawa K, Khalil A, Ishibashi T, Inomata H, Sueishi K. The relation between expression of vascular endothelial growth factor and breakdown of the blood-retinal barrier in diabetic rat retinas. *Lab Invest*. 1996;74:819–825.

39. Das A, McGuire PG, Rangasamy S. Diabetic Macular Edema: Pathophysiology and Novel Therapeutic Targets. *Ophthalmology*. 2015;122:1375–1394.

40. Kawasaki R, Bauer M, Bezlyak V, Ogura Y. Treatment patterns for retinal diseases in patients newly-treated with anti-VEGF agents: A retrospective analysis of claims data from the Japan Medical Data Center database. *Jpn J Ophthalmol*. 2021;65:215–226.

41. Chen JT, Chen LJ, Chen SN, et al. Management of diabetic macular edema: experts' consensus in Taiwan. *Jpn J Ophthalmol*. 2020;64:235–242.

42. Bressler SB, Ayala AR, Bressler NM, et al. Persistent Macular Thickening After Ranibizumab Treatment for Diabetic Macular Edema With Vision Impairment. *JAMA Ophthalmol*. 2016;134:278–285.