

Efficacy of an Anti-Semaphorin 3A Neutralizing Antibody in a Male Experimental Retinal Vein Occlusion Mouse Model

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PURPOSE. Semaphorin 3A (Sema3A) is a promising therapeutic target for macular edema in age-related macular degeneration, diabetic retinopathy, and retinal vein occlusion (RVO). Anti-vascular endothelial growth factors (anti-VEGFs) are the current standard of care for many retinal diseases. This study investigated the Sema3A neutralizing antibody BI-X and/or anti-VEGF therapy (aflibercept) in an RVO mouse model. Treatment efficacy was examined and grouped by timing subsequent to the RVO mouse model induction: efficacy against the onset of intraretinal edema 1 day postinduction and protective effects at 7 days postinduction.

METHODS. We examined the changes in expression of Sema3A in the retina of an RVO mouse model. In addition, changes in expression of tumor necrosis factor (TNF)- α and semaphorin-related proteins (neuropilin-1 and plexin A1) in the retina upon treatment were analyzed by Western blotting. The effects of BI-X and/or aflibercept were evaluated using measures of retinal edema, blood flow, and thinning of the inner nuclear layer.

RESULTS. Induction of vein occlusion in the RVO mouse model significantly increased Sema3A expression in the retina, particularly in the inner nuclear layer. BI-X was effective as a monotherapy and in combination with anti-VEGF therapy, demonstrating a beneficial effect on intraretinal edema and retinal blood flow. Moreover, in the RVO mouse model, BI-X monotherapy normalized the changes in expression of TNF- α and semaphorin-related proteins.

CONCLUSIONS. These findings support targeting Sema3A to treat intraretinal edema and retinal ischemia.

Keywords: diabetic macular ischemia, diabetic macular edema, diabetic retinopathy, retinal vein occlusion, Sema3A

A major cause of vision loss in retinal vein occlusion (RVO) and diabetic macular edema (DME) is intraretinal edema.¹⁻³ Macular edema is directly implicated in severe impairment of retinal function.^{4,5} In particular, cystoid macular edema, which is the abnormal accumulation of intraretinal fluid, leads to loss of retinal function.⁶ The predominant treatment strategy for macular edema is the use of anti-vascular endothelial growth factor (anti-VEGF) therapies. VEGF plays a key role in vascular hyperpermeability,⁷ and intraocular levels of VEGF in the eyes with RVO and DME are elevated.^{8,9} Therefore, targeting VEGF-A is a reasonable treatment strategy for intraretinal edema. However, recent studies suggest that anti-VEGF therapies might cause adverse events, such as retinal thinning,^{10,11} retinal pigment epithelial atrophy,¹² and tractional retinal detachment due to increasing fibrotic tissue,^{12,13} which can lead to irreversible vision loss. One strategy to address these problems is to develop new treatments that target factors other than VEGF-A, working either in conjunction with current treatments or as a novel monotherapy. As the presence of intraocular edema correlates with visual function,¹⁴ the development of

new effective treatment options for edema may have clinical benefits.

Semaphorin 3A (Sema3A) is part of the semaphorin superfamily of proteins and is a guidance molecule known for regulating axon formation¹⁵ and dendrite growth¹⁶ in the central nervous system. The anterior visual pathway (including the retina) is part of the central nervous system; the eyes and brain are closely connected in terms of embryology,¹⁷ anatomy,^{18,19} and physiology.²⁰ Sema3A has been shown to affect rod photoreceptor responses to injury²¹ and to promote dendritic growth of retinal ganglion cells.²²

Sema3A functions not only in the nervous system, but also in the vascular system. Sema3A binds to co-receptor neuropilin-1 (Nrp-1), which is also a co-receptor for VEGF-A.²³ VEGF-A is a well-known angiogenesis factor, whereas Sema3A exerts an anti-angiogenic effect by vasorepulsion through cytoskeletal collapse in the filopodia of endothelial tip cells,²³ maintaining physiological balance in blood vessels. Furthermore, Sema3A can induce vascular permeability similar to VEGF-A.¹² There are clinical reports indicating elevated levels of Sema3A in the aqueous humor

of eyes with RVO.²⁴ Based on this evidence, we hypothesized that inhibiting Sema3A might be effective for reducing intraretinal edema observed in patients with RVO and DME. However, the effects of Sema3A antagonism on intraretinal fluid and related retinal damage have not been explored until now.

The aim of this study was to investigate the effect of Sema3A antagonism on retinal edema and blood flow using an RVO mouse model. Antagonism of Sema3A was examined by using BI-X, a humanized monoclonal antibody that binds to Sema3A and antagonizes its biological effects.²⁵ BI-X has been shown to inhibit cytoskeletal collapse and hyperpermeability induced by Sema3A in human retinal microvascular cells.²⁵ Furthermore, BI-X increased endothelial tip cell density and reduced the avascular area in a mouse model of oxygen-induced retinopathy, demonstrating its beneficial effects in conditions of retinal ischemia.²⁵ BI-X is in clinical development in patients with diabetic macular ischemia and diabetic retinopathy treated with pan-retinal photocoagulation,²⁶ and has been shown to bind Sema3A from mice with high affinity. We investigated the effects of BI-X as a monotherapy and as combined treatment with an anti-VEGF agent (aflibercept) in an RVO mouse model. Changes in the expression of TNF- α and semaphorin-related proteins after treatment were investigated in the monotherapy and combination treatment groups.

METHODS

Animals

Female mice become sexually mature after 5 weeks of age, at which point the effects of the sexual cycle appear and are usually different for individual mice.²⁷ As it has been reported that female sex hormones could affect retinal abnormal vessels,²⁸ we selected only male mice for this study. Male albino ddY mice (8 weeks old; 35–45 g body weight) were obtained from Japan SLC (Hamamatsu, Japan). The mice were housed under a 12-hour light-dark cycle at $24 \pm 2^\circ\text{C}$ and $55 \pm 15\%$ humidity with free access to food (CLEA rodent diet CE-2; CLEA Japan, Inc., Tokyo, Japan) and water. Experimental procedures adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. We performed each experiment using five or six mice per group. In total, 200 mice were used in this study.

RVO Mouse Model

Normal mice did not develop RVO, and all normal mice had healthy eyes. Murine RVO was performed according to methods established by our laboratory.^{29,30} In brief, 8-week-old male ddY mice were anesthetized by an intramuscular injection of a mixture of ketamine (120 mg/kg; Daiichi Sankyo, Tokyo, Japan) and xylazine (6 mg/kg; Bayer HealthCare, Osaka, Japan). They then received an injection of rose bengal (8 mg/mL; Wako, Osaka, Japan) into the tail vein, followed by photocoagulation of three retinal veins using a 532 nm laser (condition of laser irradiation: 50 mW, 5 seconds, and 50 μm ; Meridian AG, Bierigustrasse, Switzerland).

Drug Administration

Under anesthesia (a mixture of 120 mg/kg ketamine [Daiichi Sankyo] and 6 mg/kg xylazine [Bayer HealthCare AG]), BI-X and/or an anti-VEGF agent (aflibercept) were injected intravitreally into the right eye of each mouse, at a dose of 10 $\mu\text{g}/\text{eye}$ and an injection volume of 2 μL . The composition of the solvent in BI-X and aflibercept is 51 mM sodium phosphate, 60 mg/mL trehalose, and 0.04% tween 20. This solvent was intravitreally administered at a volume of 2 μL to the vehicle-treated group. To examine treatment efficacy, drugs were administered either immediately (early phase) or 7 days (late phase) after laser irradiation.

Immunoblotting

Drugs were intravitreally administered to male ddY mice (8 weeks old; $n = 5$ or 6) either immediately or 7 days after laser irradiation. Mice were decapitated by cervical dislocation after successful implementation of the murine model of RVO. The eyes were enucleated, with the retinas carefully isolated and frozen at -80°C . The whole retina was homogenized in cell-lysis buffer using a homogenizer (Microtec Co., Ltd., Chiba, Japan) and centrifuged at $12,000 \times g$ for 20 minutes at 4°C . The protein content of the lysates was determined by the bicinchoninic acid (BCA) assay. Retinal proteins were separated using 5% to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient gel (Wako) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Co., Billerica, MA, USA). After blocking with Blocking One-P (Nakalai Tesque, Inc., Kyoto, Japan) at room temperature for 30 minutes, the transferred membranes were incubated with an antibody against the specific target overnight at 4°C . The primary antibodies were mouse monoclonal anti-tumor necrosis factor (TNF)- α antibody (1:100 dilution; sc-133192 [Santa Cruz]), rabbit polyclonal anti-Sema3A antibody (1:1000 dilution; ab23391 [Abcam]), rabbit monoclonal anti-Nrp-1 antibody (1:1000 dilution; ab81321 [Abcam]), and rabbit polyclonal anti-plexin A1 antibody (1:1000 dilution; ab23391 [Abcam]). After the membranes were washed with Tris-buffered saline containing 0.05% Tween 20, they were incubated with a secondary antibody, either goat anti-rabbit horseradish peroxidase (HRP)-conjugated (1:1000 dilution; #32460 [Thermo Fisher Scientific]) or goat anti-mouse HRP-conjugated (1:1000 dilution; #32430 [Thermo Fisher Scientific]). The immunoreactive bands were visualized using Immuno Star LD (Wako), and the band intensity in the membranes was measured by LAS-4000 Luminescent Image Analyzer (Fuji Film Co. Ltd., Tokyo, Japan).

Immunostaining

Drugs were intravitreally administered to male ddY mice (8 weeks old; $n = 5$) immediately after laser irradiation and the eyes were enucleated. Whole eyes were fixed in 4% paraformaldehyde (PFA) for 48 hours, transferred into 5, 10, 15, and 20% sucrose every 3 hours, and then placed in 25% sucrose for 24 hours at 4°C . The eyes were embedded in optimal cutting temperature (OCT) compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and sectioned (thickness = 10 μm) using a cryostat (Leica microsystems, Co., Ltd., Tokyo, Japan). The sections were blocked in phosphate buffer solution (PBS) containing 10% normal goat serum (Vector Labs, Burlingame, CA, USA) for 1 hour at room

temperature, and then incubated overnight with primary antibodies (rabbit polyclonal anti-sema3A antibody [1:50 dilution; ab23391 [Abcam]) at 4°C. After being washed with PBS, the sections were incubated with the secondary antibody (Alexa Fluor488 goat anti-rabbit IgG [Thermo Fisher Scientific]) for 1 hour at room temperature. The nuclei were counterstained with Hoechst 33342 (Thermo Fisher Scientific) for 10 minutes. The negative control was incubated without primary antibodies. The images of stained sections were obtained using a fluorescence microscope (BZ-710; Keyence, Osaka, Japan); the fluorescence intensity of the inner nuclear layer (INL) was quantified in the retina at 500 μm from the optic nerve head.

Histological Analyses

Drugs were administered to male ddY mice (8 weeks old; $n = 5$) either immediately or 7 days after laser irradiation and the eyes were enucleated. Eyeballs were fixed in 4% PFA (Wako) with 0.1 M PBS (PB; pH 7.4) for 48 hours at 4°C. Paraffin-embedded tissue (thickness = 5 μm) using microtome (Leica Microsystems, Co., Ltd.) was stained with hematoxylin and eosin. The stained sections were imaged with the “All-in-One” BZ-X710 fluorescent microscope (Keyence, Osaka, Japan). The INL thickness was measured every 240 μm from the optic nerve head using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Measurements of Ocular Blood Flow and Retinal Nonperfused Areas

Drugs were administered to male ddY mice (8 weeks old; $n = 5$) either immediately or 7 days after laser irradiation. Under the anesthesia using a mixture of 120 mg/kg ketamine (Daiichi Sankyo) and 6 mg/kg xylazine (Bayer HealthCare AG), mean blur rate images (an index of the relative blood flow velocity) were acquired continuously using a laser speckle flowgraphy device (LSFG; Softcare Co., Ltd) at a rate of 30 fps over a time period of approximately 4 seconds. The measured fundus area was approximately 3.8 \times 3.0 mm (width \times height), with an estimated tissue penetration of 0.5 to 1.0 mm. After the image acquisition, the vessel and tissue areas on the optic nerve head area were automatically detected by the LSFG Analyzer software (version 3.1.14.0; Softcare Co., Ltd.) using the vessel extraction function. Mice were injected with PBS containing fluorescein-conjugated dextran (20 mg/mL; fluorescein isothiocyanate dextran; MW = 2000 kDa; Sigma-Aldrich) into the tail vein. After 5 minutes, mice were euthanized by cervical dislocation, and the eyes were enucleated. The eyes were fixed in 4% PFA for 7 hours and then the retinas were isolated. The isolated retinas were flat-mounted using Fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA). The images of flat-mounted retinas were captured with Metamorph (Universal Imaging Corp., Downingtown, PA, USA) and the size of retinal nonperfused areas was measured using the ImageJ processing software.

Statistical Analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 J for Windows (Japan Inc., Tokyo, Japan). Comparisons between

group means were performed using the Student's *t*-test and 1-way ANOVA, followed by the Dunnett's test and Tukey's test. All results are presented as mean \pm SEM, and $P < 0.05$ was considered significant.

RESULTS

The Expression of Sema3A and Nrp-1 was Increased in the Retina of an RVO Mouse Model

The expression levels of Sema3A in the retina were significantly increased at 1, 3, and 7 days after vein occlusion compared with untreated (normal) mice ($P < 0.05$; Fig. 1A). TNF- α expression levels in the retina were significantly increased at 0.5, 1, 3, and 7 days after vessel occlusion ($P < 0.05$; Fig. 1B). Levels of Nrp-1 in the retina were significantly increased at 0.5, 1, and 7 days after vessel occlusion ($P < 0.05$; Fig. 1C). Plexin A1 in the retina expression levels were significantly increased at 1 and 7 days after vessel occlusion ($P < 0.05$; Fig. 1D).

Immunostaining was performed to determine the location of Sema3A in the retinas of the RVO mouse model. Sema3A was increased in all layers in the retina of the RVO mouse model (Fig. 1E). On or after 1 day of RVO induction, the fluorescent intensity of Sema3A (green) was increased in all retinal layers, including the ganglion cell layer and the INL where the retinal vessels are located. The expression of Sema3A was significantly increased in the INL ($P < 0.05$; Fig. 1F).

BI-X Ameliorated Cystoid Edema and Decreased Retinal Blood Flow in the Early Phase of an RVO Mouse Model

The thickness of the INL was markedly increased at 1 day after laser irradiation. This increase was suppressed in both the BI-X and aflibercept monotherapy groups, as well as the BI-X + aflibercept combination group (Fig. 2A). In the vehicle-treated group, retinal blood flow was significantly reduced at 1 day after laser irradiation ($P < 0.01$). The decrease in blood flow was significantly ameliorated after administration of either BI-X ($P = 0.002$) or aflibercept monotherapy ($P = 0.018$); this improvement was significantly greater in the BI-X + aflibercept combination group than either monotherapy group ($P < 0.05$; Fig. 2B). Administration of BI-X, aflibercept, or the combination of BI-X + aflibercept immediately after laser irradiation led to a significant reduction in the size of the retinal nonperfused areas 1 day after laser irradiation compared with the vehicle-treated group ($P < 0.01$; Fig. 2C).

The Expression of Sema3A, TNF- α , and Sema3A-Related Proteins (Nrp-1 and Plexin A1) was Decreased by BI-X in the Early Phase of an RVO Mouse Model

The expression of Sema3A in the retina was significantly decreased by early injection of BI-X monotherapy and the BI-X + aflibercept combination ($P < 0.05$; Fig. 3A). The expression of TNF- α in the retina was increased in the vehicle-treated group 1 day after vessel occlusion. Early injection of BI-X or aflibercept monotherapy and BI-X + aflibercept combination treatment decreased TNF- α expression ($P < 0.05$; Fig. 3B). The expression of Nrp-1 and

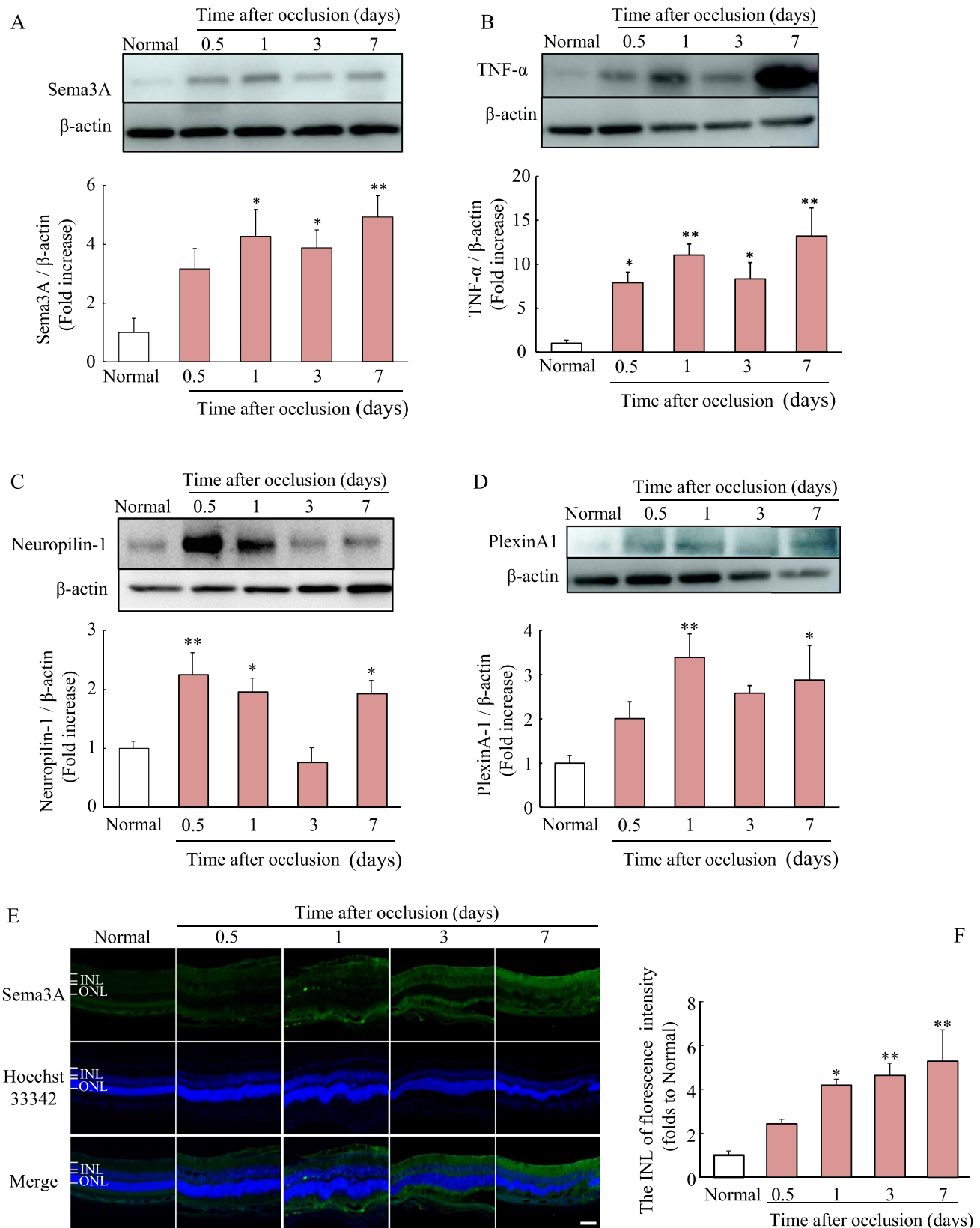


FIGURE 1. The expression of Sema3A, TNF- α , Nrp-1, and plexin A1 in the retina of an RVO mouse model. The expression levels of Sema3A (A), TNF- α (B), semaphorin-related protein Nrp-1 (C), and plexin A1 (D) in the retina at 0.5, 1, 3, and 7 days after the onset of RVO without treatment (normal). The same samples were used in the experiments shown in (A–D). (E) Representative images showing Sema3A (green) in the retinas of mice (normal) and in an RVO mouse model at 0.5, 1, 3, and 7 days after laser irradiation. The nuclei are stained blue by Hoechst 33342. (F) The expression of Sema3A was increased in the INL of the retina in the RVO mouse model. Data are shown as mean \pm SEM ($n = 5$). * $P < 0.05$, ** $P < 0.01$ versus normal (1-way ANOVA followed by Dunnett's test). The scale bar indicates 100 μ m. INL, inner nuclear layer; ONL, outer nuclear layer.

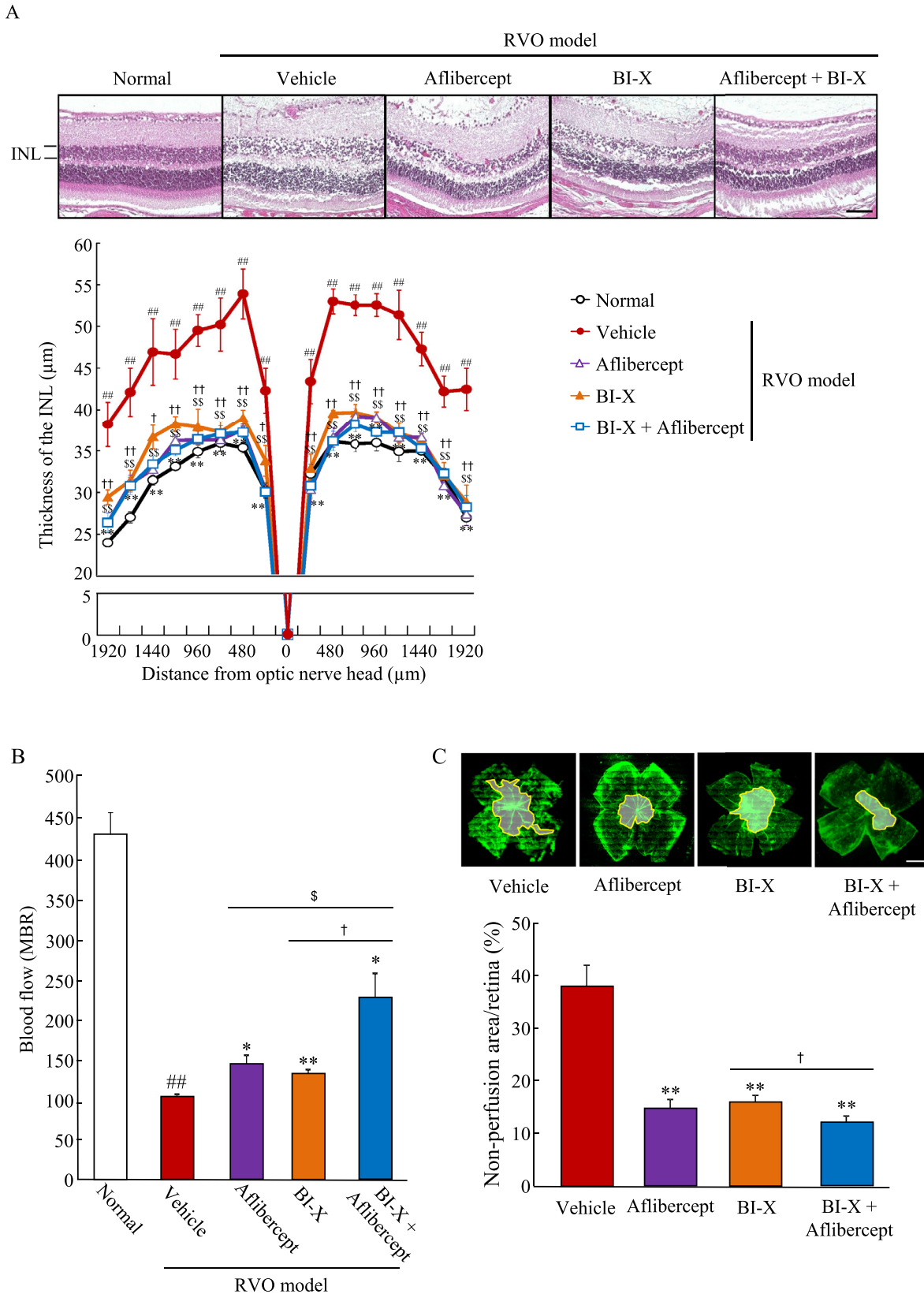


FIGURE 2. The effects of Sema3A neutralizing antibody for intraretinal fluid and retinal blood flow in the early phase of RVO model mice. BI-X and/or aflibercept was intravitreally injected immediately after laser irradiation in RVO model mice. The retinas were examined at 1 day after the administration of each treatment. (A) Representative images of retinal sections stained with hematoxylin and eosin in the normal, vehicle-treated, and BI-X and/or aflibercept-treated groups. It shows the images at 500 µm from the optic nerve head. Graphs showing the thickness of the INL. This increase was suppressed by the administration of BI-X monotherapy and BI-X + aflibercept combination treatment.

Data are shown as mean \pm SEM ($n = 5$). $^{**}P < 0.01$ (normal versus vehicle), $^{*}P < 0.05$, $^{**}P < 0.01$ (vehicle versus aflibercept), $^{††}P < 0.01$ (vehicle versus BI-X), $^{§§}P < 0.01$ (vehicle versus BI-X + aflibercept; 1-way ANOVA followed by Tukey's test). The scale bar indicates 100 μm . (B) The ocular blood flow was determined by laser speckle flowgraphy 1 day after the intravitreal injection of BI-X and/or aflibercept immediately after laser irradiation in RVO mice. $^{**}P < 0.01$ (normal versus vehicle), $^{*}P < 0.05$, $^{**}P < 0.01$ (vehicle versus aflibercept, BI-X or BI-X + aflibercept), $^{§}P < 0.05$ (aflibercept versus BI-X + aflibercept), $^{†}P < 0.05$ (BI-X versus BI-X + aflibercept; 1-way ANOVA followed by Student's *t*-test). (C) Representative images of flat-mounted retinas of vehicle and BI-X monotherapy and/or aflibercept monotherapy groups. Graphs showing the size of the nonperfused areas. Data are shown as mean \pm SEM ($n = 5$). $^{**}P < 0.01$ (versus vehicle) $^{†}P < 0.05$ (BI-X versus BI-X + aflibercept; 1-way ANOVA followed by Student's *t*-test). The same samples were used in the experiments shown in (B) and (C). The scale bar indicates 500 μm .

plexin A1 in the retina were also increased in the vehicle-treated group 1 day after the vein occlusion. Nrp-1 expression in the retina was significantly decreased by early injection of the BI-X + aflibercept combination treatment ($P < 0.05$; Fig. 3C). Furthermore, the expression of plexin A1 in the retina was significantly decreased by the early injection of BI-X monotherapy and BI-X + aflibercept combination ($P < 0.05$; Fig. 3D).

BI-X Ameliorated Retinal Thinning and Retinal Blood Flow in the Late Phase of an RVO Mouse Model

The thickness of the INL was significantly decreased at 8 days after laser irradiation in the vehicle-treated group ($P < 0.01$). The administration of aflibercept monotherapy accelerated the extent of retinal thinning. However, retinal thinning was suppressed by the intravitreal injection of BI-X monotherapy at 7 days after laser irradiation. Importantly, retinal thinning was also significantly suppressed in the BI-X + aflibercept combination group compared with the aflibercept monotherapy group, albeit less than with BI-X monotherapy ($P < 0.05$ and $P < 0.01$, respectively; Fig. 4A).

The effect of BI-X and/or aflibercept on ocular blood flow 7 days after laser irradiation was also examined. Retinal blood flow was significantly reduced 8 days after laser irradiation in the vehicle-treated group ($P < 0.01$). Aflibercept monotherapy significantly reduced retinal blood flow relative to the vehicle-treated group ($P < 0.01$), whereas BI-X monotherapy significantly improved retinal blood flow ($P < 0.05$; Fig. 4B). The decrease in retinal blood flow was also significantly suppressed in the BI-X + aflibercept combination group compared with the aflibercept monotherapy group ($P < 0.05$; Fig. 4B), albeit less than with BI-X monotherapy.

The size of the retinal nonperfused area was increased by the administration of aflibercept monotherapy at 7 days after laser irradiation but was significantly reduced after administration of BI-X monotherapy compared with the vehicle-treated group ($P < 0.01$). Combination treatment with BI-X + aflibercept also reduced the size of the retinal nonperfused area, but to a lesser extent than treatment with BI-X monotherapy (Fig. 4C).

The Expression of Sema3A, TNF- α , and Sema3A-Related Proteins (Nrp-1 and Plexin A1) was Decreased by BI-X in the Late Phase of an RVO Mouse Model

The expression of Sema3A in the retina was significantly decreased by early injection of BI-X monotherapy and by the BI-X + aflibercept combination ($P < 0.05$; Fig. 5A). The expression of TNF- α in the retina was increased in

the vehicle-treated group 8 days after the vessel occlusion, and was not changed by the late injection of aflibercept monotherapy or BI-X + aflibercept combination treatment compared with the vehicle-treated group. However, the administration of BI-X monotherapy significantly reduced the retinal expression of TNF- α in the late phase ($P < 0.01$). In addition, compared with the aflibercept monotherapy group, the retinal expression of TNF- α was significantly reduced by the administration of the BI-X + aflibercept combination ($P < 0.01$; see Fig. 5A). The retinal expression of Nrp-1 and plexin A1 was increased in the vehicle-treated group 8 days after the vessel occlusion and was not changed by the administration of aflibercept monotherapy compared with the vehicle-treated group. Compared with the aflibercept monotherapy group, the retinal expression of Nrp-1 was significantly reduced by the administration of BI-X + aflibercept combination treatment ($P < 0.05$; Fig. 5B). Notably, the administration of BI-X significantly decreased the retinal expression levels of Nrp-1 and plexin A1 in the late phase (both $P < 0.01$; see Figs. 5B, 5C).

DISCUSSION

In a previous study, we demonstrated that the hyperpermeability induced by Sema3A and VEGF-A in human retinal microvascular cells is additive, and that BI-X inhibits only the hyperpermeability evoked by Sema3A. Although Sema3A is known to induce retinal vascular hyperpermeability,³¹ there are currently no treatments that clinically target Sema3A. Here, we demonstrate that Sema3A antagonism can suppress the blood-retinal barrier dysfunction associated with RVO via the suppression of inflammation. Using an RVO mouse model, we have demonstrated the accumulation of Sema3A in the retina and the efficacy of BI-X against edema and hypoperfusion. Furthermore, we showed that Sema3A in the retina can regulate the expression of TNF- α , a proinflammatory cytokine.

Upregulation of Sema3A has been previously reported in the retina, as well as in the brain and heart, when tissues become ischemic.³²⁻³⁴ Our data show that expression of Sema3A in the retina of an RVO mouse model increases in a time-dependent manner, until 7 days after vascular occlusion (see Fig. 1A). Because the retina of the RVO mouse model is ischemic,³⁰ the increase in the retinal expression of Sema3A in this model correlates with previous reports.³⁴⁻³⁶ TNF- α , Nrp-1, and plexin A1, co-receptors for Sema3A, were also increased in the retina of the RVO mouse model in a biphasic manner (see Figs. 1B-D). The expression of inflammation-related factors, such as IL-6 and ICAM1, in an RVO mouse model has also shown biphasic changes similar to that of Nrp-1 and plexin A1.³⁰ Although it is possible that the retinal expression of Nrp-1 and plexin A1 is associated with proinflammatory signals, the precise mechanism of this biphasic change will need further investigation.

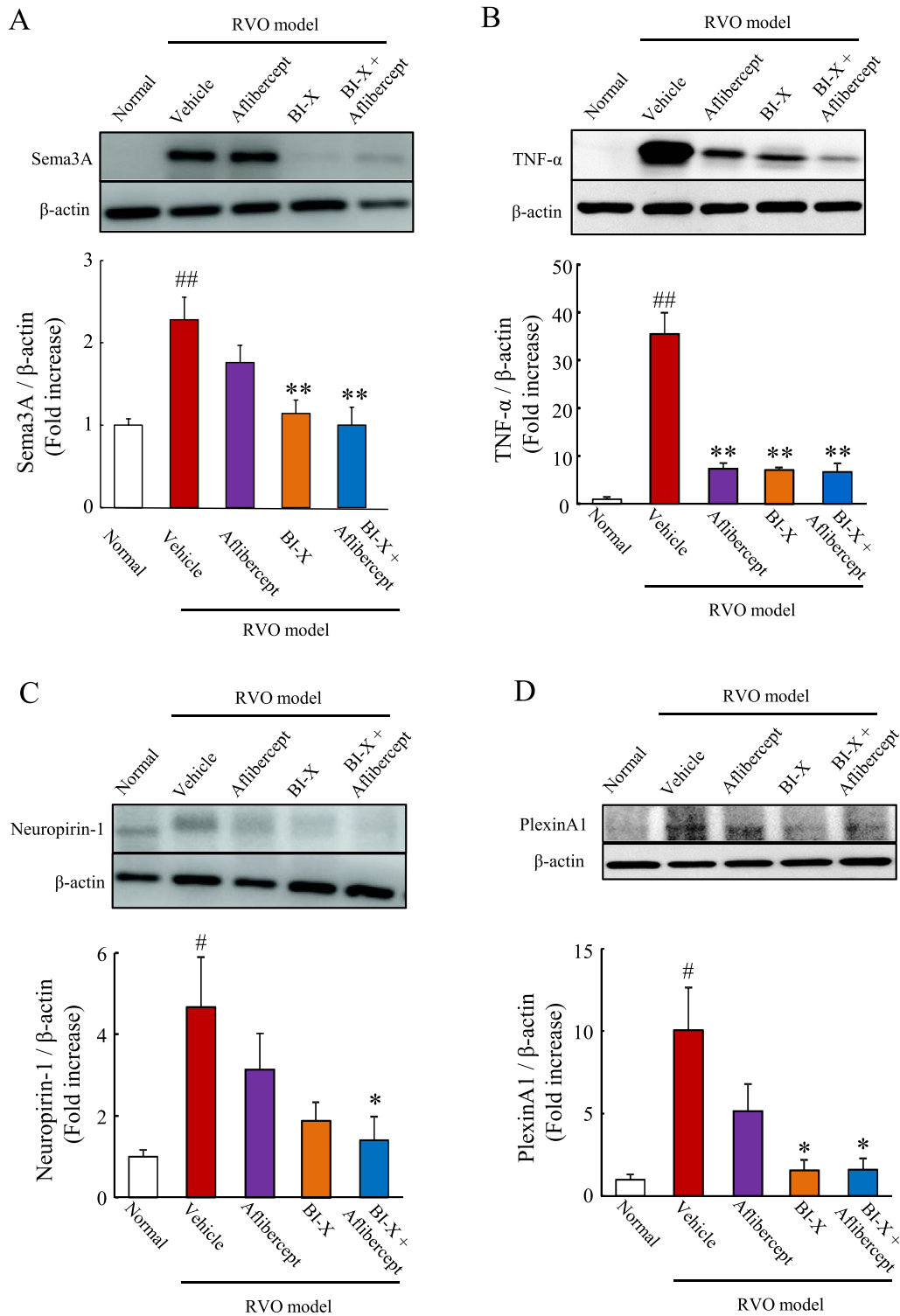


FIGURE 3. The effects of Sema3A neutralizing antibody on Sema3A, TNF- α , and semaphorin-related proteins in the early phase of RVO model mice. The RVO mice were injected intravitreally with BI-X and/or aflibercept immediately after vessel occlusion. The retinas were examined at 1 day after injection of BI-X and/or aflibercept. Representative Western blots showing the expression of Sema3A, TNF- α , Nrp-1, and plexin A1 after the intravitreal injection of BI-X and/or aflibercept immediately following adiponectin antibody. Quantitative analysis of Sema3A, TNF- α , Nrp-1, and plexin A1 with normalization to β -actin. Aflibercept monotherapy, BI-X monotherapy, and BI-X + aflibercept combination treatment significantly decreased the retinal expression of Sema3A (A), TNF- α (B), Nrp-1 (C), and plexin A1 (D) in the early phase. Data are shown as mean \pm SEM ($n = 6$). ^{*} $P < 0.05$, ^{**} $P < 0.01$ (versus normal), [#] $P < 0.05$, ^{##} $P < 0.01$ (versus vehicle; 1-way ANOVA followed by Student's t -test). The same samples were used in all experiments (A–D).

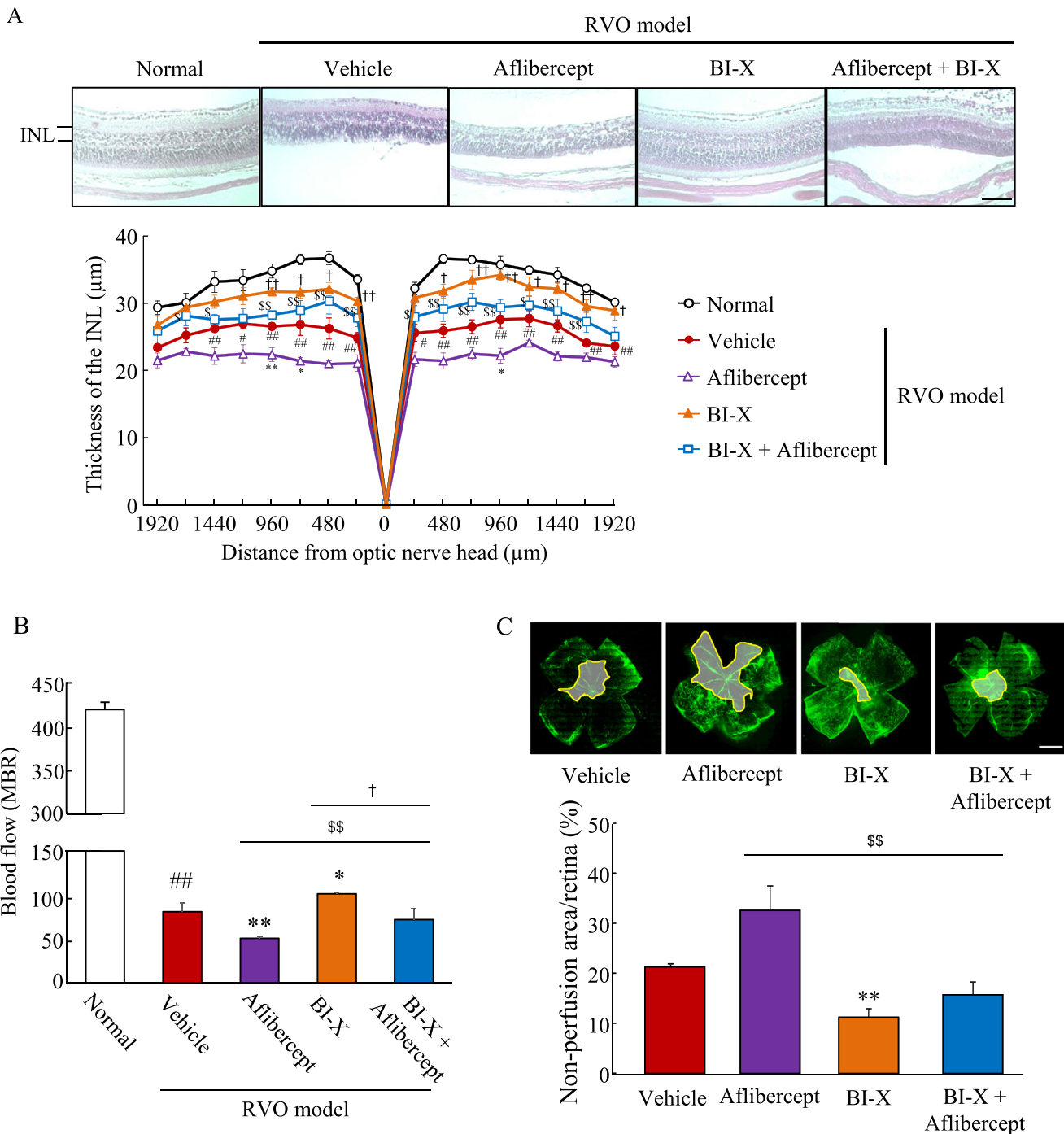


FIGURE 4. The effects of Sema3A neutralizing antibody for intraretinal fluid and retinal blood flow in the late phase of RVO model mice. BI-X and/or aflibercept was intravitreally injected 7 days after laser irradiation. **(A)** Representative images of retinal sections stained with hematoxylin and eosin in the normal, vehicle-treated, and BI-X monotherapy and/or aflibercept monotherapy groups, at 500 µm from the optic nerve head. Graphs show the thickness of the INL. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (normal versus vehicle), $^*P < 0.05$, $^{**}P < 0.01$ (vehicle versus aflibercept), $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ (vehicle versus BI-X), $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ (aflibercept versus BI-X + aflibercept; 1-way ANOVA followed by Tukey's test). The scale bar indicates 100 µm. **(B)** The area of nonperfusion was determined by laser speckle flowgraphy 1 day after the intravitreal injection of BI-X and/or aflibercept, 7 days after laser irradiation. $^{\#\#}P < 0.01$ (versus normal), $^*P < 0.05$, $^{**}P < 0.01$ (versus vehicle), $^{\$}P < 0.05$ (aflibercept versus BI-X + aflibercept), $^{\dagger\dagger}P < 0.01$ (BI-X versus BI-X + aflibercept; 1-way ANOVA followed by Student's *t*-test). **(C)** The size of retinal nonperfused areas were increased by the administration of aflibercept monotherapy in the late phase, but were reduced by BI-X monotherapy compared to the vehicle-treated group. Data are shown as mean \pm SEM ($n = 5$). $^{**}P < 0.01$ (versus vehicle), $^{\$}P < 0.05$ (aflibercept versus BI-X + aflibercept; 1-way ANOVA followed by Student's *t*-test). The same samples were used in the experiments shown in **(B)** and **(C)**. The scale bar indicates 500 µm.

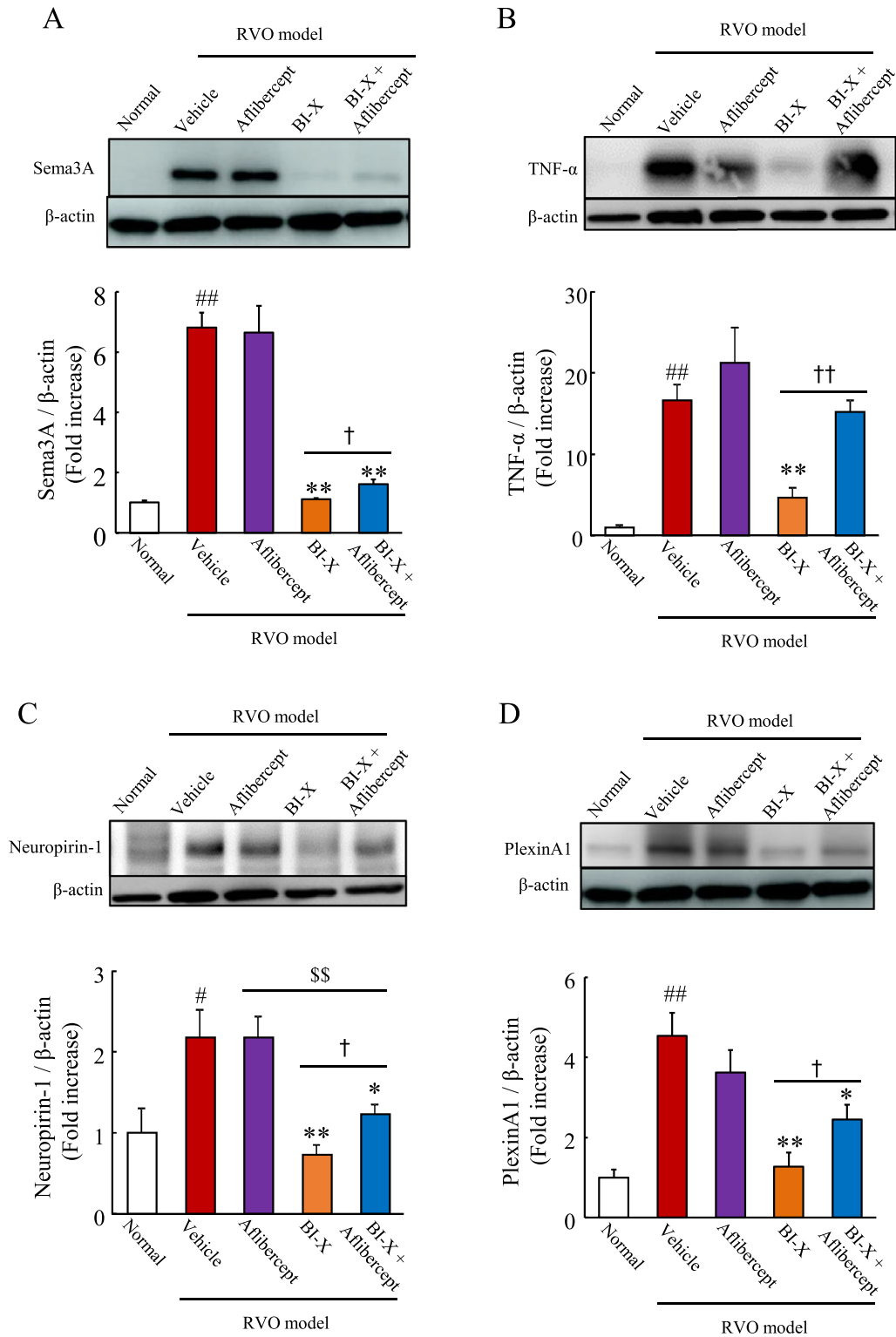


FIGURE 5. The effects of Sema3A neutralizing antibody on Sema3A, TNF- α , and semaphorin-related protein in the late phase of RVO model mice. The RVO mice were injected intravitreally with BI-X and/or aflibercept 7 days after the vessel occlusion. The retinas were examined 8 days after injection of BI-X and/or aflibercept. Representative Western blots showing the expression of Sema3A, TNF- α , Nrp-1, and plexin A1 after the intravitreal injection of BI-X and/or aflibercept immediately following adiponectin antibody. Quantitative analysis of Sema3A, TNF- α , Nrp-1, and plexin A1 with normalization to β -actin. Aflibercept treatment was not changed compared with the vehicle-treated group. In the late phase, the administration of BI-X monotherapy decreased the expression levels of Sema3A (A), TNF- α (B), Nrp-1 (C), and (D). Data are shown as mean \pm SEM ($n = 6$). [#] $P < 0.05$, ^{**} $P < 0.01$ (versus normal), ^{*} $P < 0.05$, ^{**} $P < 0.01$ (vehicle versus BI-X), ^{\$\$} $P < 0.01$ (aflibercept versus BI-X + aflibercept), [†] $P < 0.05$, ^{††} $P < 0.01$ (BI-X versus BI-X + aflibercept; 1-way ANOVA followed by Student's *t*-test). The same samples were used in all experiments (A–D).

We also examined the localization of Sema3A in the retina of the RVO mouse model. After 1 day of vascular occlusion, the expression of Sema3A increased in all retinal layers, especially in the INL where retinal deep capillaries exist (see Figs. 1C, 1D). This is similar to the localization of Sema3A in the retina of streptozotocin-induced diabetic mice with retinal vascular hyperpermeability.³¹ These findings indicate that Sema3A expression is closely related to the increased permeability of deep retinal vessels in an RVO mouse model.

Administration of BI-X following RVO markedly suppressed retinal edema (see Fig. 2A). Cerani et al. reported that Sema3A is increased in the vitreous of patients with DME, and that Sema3A induces retinal vascular permeability.³¹ Thus, Sema3A is one of the factors that contributes to intraretinal edema. The data presented in this study indicate that Sema3A antagonism may be an effective treatment for intraretinal edema. In addition, BI-X may have an inhibitory effect on reduced blood flow in retinal vessels (see Figs. 2C, 4C). Recently, it has been reported that inhibition of Sema3E/plexin D1 signaling in a model of middle cerebral artery occlusion can inhibit the cerebral blood flow reduction. The fact that levels of Sema3A are markedly increased in diabetic retinopathy with retinal ischemia suggests that Sema3A may play a pivotal role in blood flow reduction in the retina; the results presented here are consistent with this hypothesis. Notably, the BI-X + aflibercept combination treatment group showed an additional increase in retinal blood flow compared with aflibercept monotherapy. BI-X + aflibercept combination treatment may be more effective than aflibercept monotherapy in treating retinal ischemia (see Figs. 2C, 2D, 4C, 4D). Early administration of both BI-X and aflibercept significantly decreased the expression of TNF- α , and the expression of Nrp-1 and plexin A1 in the retina approached normal levels after BI-X monotherapy (see Fig. 3). These data indicate that BI-X + aflibercept combination treatment could ameliorate inflammation, and that BI-X monotherapy may be suitable for suppressing the activation of Nrp-1- and plexin A1-mediated signaling in the retina after RVO.

Importantly, BI-X monotherapy improved retinal thinning in the late stage after RVO, whereas aflibercept monotherapy resulted in progressive thinning of the retina. The INL was also significantly thicker in the BI-X + aflibercept combination group than in the aflibercept monotherapy group (see Fig. 4A). These results suggest that antagonizing Sema3A could have neuroprotective effects in the INL and on the pathology of RVO. In the late phase of this RVO mouse model, the level of VEGF in the retina had normalized³⁰; under such conditions, a Sema3A antagonist treatment for retinal neuroprotection may be advantageous over an anti-VEGF therapy. It has been previously reported that Sema3A neutralizing antibodies have a protective effect on the retinal injury associated with optic nerve axotomy.³⁵ The Sema3A inhibitor SM-345431 has also been reported to have a protective effect on the corneal epithelium.³⁶ These previous reports are consistent with the results presented here and imply that elevated Sema3A levels may be involved in retinal pathology.

In addition, as in the early phase of the RVO mouse model, BI-X inhibited the decrease in blood flow in the late phase. In the late phase, aflibercept monotherapy exacerbated blood flow reduction, whereas BI-X monotherapy suppressed the reduction. As the retina (like other tissues) is nourished through systemic blood flow, neuroprotection is potentially achieved by the amelioration of retinal blood flow. At 8 days after occlusion in the

retina of the RVO mouse model, TNF- α , Nrp-1, and plexin A1 levels in the retina were notably increased and hardly changed by aflibercept monotherapy; however, all were markedly reduced by BI-X monotherapy (see Fig. 5). Aflibercept reduced the retinal expression of TNF- α in the early phase of the RVO mouse model (see Fig. 3B) but had no effect on the retinal expression of TNF- α in the late phase of the RVO mouse model (see Fig. 5B). Interestingly, the Sema3A neutralizing antibody BI-X reduced the retinal expression of Sema3A and TNF- α in both the early and late phases of the RVO mouse model (see Fig. 3A, Fig. 5A). The biphasic reduction of TNF- α expression in the retina observed with BI-X is consistent with the report that TNF- α initiates not only inflammation and response to injury, but also subsequent epidermal repair.³⁷ These complex effects have also been determined from the involvement of multiple second-messenger pathways of TNF- α signaling.³⁸ The most commonly reported role of TNF- α in the eye is to induce cell death.³⁹ In other words, the results of this study indicate that Sema3A neutralizing antibodies may inhibit retinal cell death at any time point during RVO pathology. Direct interactions between Nrp-1 and Fer kinase, which is selectively associated with the NRP1 cytoplasmic domain, have been reported to be important for Sema3A-induced neuronal death,³⁴ supporting our data on the induction of neuronal cell death through Nrp-1. These results suggest that neutralizing Sema3A could inhibit retinal thinning in the RVO mouse model through inactivation of Sema3A-induced, plexin A1-mediated signaling, and reduce the retinal inflammation.

The results of this study provide evidence that the Sema3A neutralizing antibody BI-X is effective at treating retinal edema and retinal tissue thinning not only as a monotherapy but also in combination with an anti-VEGF agent. This discovery could shed light on the understanding of RVO pathogenesis, including macular edema and retinal neuropathy, and may aid the development of new treatment options for patients with RVO. Furthermore, these findings expand our understanding of the function of Sema3A and may provide a new treatment option for patients with macular edema who have not sufficiently responded to anti-VEGF therapy alone.

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References

- McIntosh RL, Rogers SL, Lim L, et al. Natural history of central retinal vein occlusion: an evidence-based systematic review. *Ophthalmology*. 2010;117:1113–1123.e1115.
- Rogers SL, McIntosh RL, Lim L, et al. Natural history of branch retinal vein occlusion: an evidence-based systematic review. *Ophthalmology*. 2010;117:1094–1101.e1095.
- Patz A, Schatz H, Berkow JW, Gittelsohn AM, Ticho U. Macular edema—an overlooked complication of diabetic retinopathy. *Trans Am Acad Ophthalmol Otolaryngol*. 1973;77:OP34–OP42.

4. Ogino K, Tsujikawa A, Nakamura H, et al. Focal macular electroretinogram in macular edema secondary to central retinal vein occlusion. *Invest Ophthalmol Vis Sci.* 2011;52:3514–3520.
5. Sugar EA, Jabs DA, Altaweel MM, et al. Identifying a clinically meaningful threshold for change in uveitic macular edema evaluated by optical coherence tomography. *Am J Ophthalmol.* 2011;152:1044–1052.e1045.
6. Murakami T, Yoshimura N. Structural changes in individual retinal layers in diabetic macular edema. *J Diabetes Res.* 2013;2013:920713.
7. Dvorak HF, Sioussat TM, Brown LF, et al. Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. *J Exp Med.* 1991;174:1275–1278.
8. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med.* 1994;331:1480–1487.
9. Noma H, Funatsu H, Yamasaki M, et al. Pathogenesis of macular edema with branch retinal vein occlusion and intraocular levels of vascular endothelial growth factor and interleukin-6. *Am J Ophthalmol.* 2005;140:256–261.
10. Toth CA, Tai V, Pistilli M, et al. Distribution of OCT features within areas of macular atrophy or scar after 2 years of anti-VEGF treatment for neovascular AMD in CATT. *Ophthalmol Retina.* 2019;3:316–325.
11. Du J, Patrie JT, Prum BE, Netland PA, Shildkrot YE. Effects of intravitreal anti-VEGF therapy on glaucoma-like progression in susceptible eyes. *J Glaucoma.* 2019;28:1035–1040.
12. Arevalo JF, Maia M, Flynn HW, Jr., et al. Tractional retinal detachment following intravitreal bevacizumab (Avastin) in patients with severe proliferative diabetic retinopathy. *Br J Ophthalmol.* 2008;92:213–216.
13. Yonekawa Y, Wu WC, Nitulescu CE, et al. Progressive retinal detachment in infants with retinopathy of prematurity treated with intravitreal bevacizumab or ranibizumab. *Retina.* 2018;38:1079–1083.
14. Ogino K, Tsujikawa A, Murakami T, et al. Evaluation of macular function using focal macular electroretinography in eyes with macular edema associated with branch retinal vein occlusion. *Invest Ophthalmol Vis Sci.* 2011;52:8047–8055.
15. Messersmith EK, Leonardo ED, Shatz CJ, Tessier-Lavigne M, Goodman CS, Kolodkin AL. Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron.* 1995;14:949–959.
16. Shelly M, Cancedda L, Lim BK, et al. Semaphorin3A regulates neuronal polarization by suppressing axon formation and promoting dendrite growth. *Neuron.* 2011;71:433–446.
17. Jacobson M, Hirose G. Origin of the retina from both sides of the embryonic brain: a contribution to the problem of crossing at the optic chiasma. *Science.* 1978;202:637–639.
18. Oksche A. Evolution of the pineal complex: correlation of structure and function. *Ophthalmic Res.* 1984;16:88–95.
19. Coleman JE, Law K, Bear MF. Anatomical origins of ocular dominance in mouse primary visual cortex. *Neuroscience.* 2009;161:561–571.
20. Schmidt TM, Chen SK, Hattar S. Intrinsically photosensitive retinal ganglion cells: many subtypes, diverse functions. *Trends Neurosci.* 2011;34:572–580.
21. Kung F, Wang W, Tran TS, Townes-Anderson E. Sema3A reduces sprouting of adult rod photoreceptors in vitro. *Invest Ophthalmol Vis Sci.* 2017;58:4318–4331.
22. Chan-Juan H, Sen L, Li-Qianyu A, Jian Y, Rong-Di Y. MicroRNA-30b regulates the polarity of retinal ganglion cells by inhibiting semaphorin-3A. *Mol Vis.* 2019;25:722–730.
23. Miao HQ, Soker S, Feiner L, Alonso JL, Raper JA, Klagsbrun M. Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. *J Cell Biol.* 1999;146:233–242.
24. Guo S, Ren J, Li Z, Fan X, Qin L, Li J. Aqueous semaphorin 3A level correlates with retinal macular oedema and ganglion cell degeneration in patients with retinal vein occlusion. *Acta Ophthalmol.* 2019;97:273–278.
25. Thomas L, Zippel N, Hess Kenny C, Wu H, Garneau M, Bakker RA. In vitro and in vivo studies of BI-X: a humanised Sema3A antibody. *Invest Ophthalmol Vis Sci.* 2021;62:1160.
26. NCT04424290 HORNBILL: A study to test different doses of BI 764524 in patients who have had laser treatment for a type of diabetic eye disease called diabetic retinopathy with diabetic macular ischemia. Available at: <https://ClinicalTrials.gov/show/NCT04424290>.
27. Nelson JF, Karelus K, Felicio LS, Johnson TE. Genetic influences on the timing of puberty in mice. *Biol Reprod.* 1990;42:649–655.
28. Zhang H, Wang X, Xu K, et al. 17 β -estradiol ameliorates oxygen-induced retinopathy in the early hyperoxic phase. *Biochem Biophys Res Commun.* 2015;457:700–705.
29. Nishinaka A, Inoue Y, Fuma S, et al. Pathophysiological role of VEGF on retinal edema and nonperfused areas in mouse eyes with retinal vein occlusion. *Invest Ophthalmol Vis Sci.* 2018;59:4701–4713.
30. Fuma S, Nishinaka A, Inoue Y, et al. A pharmacological approach in newly established retinal vein occlusion model. *Sci Rep.* 2017;7:43509.
31. Cerani A, Tetreault N, Menard C, et al. Neuron-derived semaphorin 3A is an early inducer of vascular permeability in diabetic retinopathy via neuropilin-1. *Cell Metab.* 2013;18:505–518.
32. Ieda M, Fukuda K. New aspects for the treatment of cardiac diseases based on the diversity of functional controls on cardiac muscles: the regulatory mechanisms of cardiac innervation and their critical roles in cardiac performance. *J Pharmacol Sci.* 2009;109:348–353.
33. Hou ST, Keklikian A, Slinn J, O'Hare M, Jiang SX, Aylsworth A. Sustained up-regulation of semaphorin 3A, Neuropilin1, and doublecortin expression in ischemic mouse brain during long-term recovery. *Biochem Biophys Res Commun.* 2008;367:109–115.
34. Jiang SX, Whitehead S, Aylsworth A, et al. Neuropilin 1 directly interacts with Fer kinase to mediate semaphorin 3A-induced death of cortical neurons. *J Biol Chem.* 2010;285:9908–9918.
35. Shirvan A, Kimron M, Holdengreber V, et al. Anti-semaphorin 3A antibodies rescue retinal ganglion cells from cell death following optic nerve axotomy. *J Biol Chem.* 2002;277:49799–49807.
36. Yamazaki R, Yamazoe K, Yoshida S, et al. The Semaphorin 3A inhibitor SM-345431 preserves corneal nerve and epithelial integrity in a murine dry eye model. *Sci Rep.* 2017;7:15584.
37. Banno T, Gazel A, Blumenberg M. Effects of tumor necrosis factor-alpha (TNF alpha) in epidermal keratinocytes revealed using global transcriptional profiling. *J Biol Chem.* 2004;279:32633–32642.
38. Dbaibo GS, Obeid LM, Hannun YA. Tumor necrosis factor-alpha (TNF-alpha) signal transduction through ceramide. Dissociation of growth inhibitory effects of TNF-alpha from activation of nuclear factor-kappa B. *J Biol Chem.* 1993;268:17762–17766.
39. Tezel G, Li LY, Patil RV, Wax MB. TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes. *Invest Ophthalmol Vis Sci.* 2001;42:1787–1794.