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Angiopoietin-like protein 2 mediates endotoxin-induced acute inflammation in the eye

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ABSTRACT

Angiopoietin-like protein (Angptl) 2 is a key mediator linking obesity to chronic adipose-tissue inflammation and systemic insulin resistance, and increasing evidence has shown that Angptl2 is associated with various chronic inflammatory diseases such as cancer and dermatomyositis; however, it remains unclear that Angptl2 functions in acute inflammation. In this study, we investigate whether Angptl2 plays a role in acute inflammation in the eye with endotoxin-induced uveitis (EIU). Angptl2 was widely expressed in the normal mouse retina, while Angptl2−/− mice did not exhibit any changes in retinal cell marker expression and morphological analyses. Treatment with lipopolysaccharide (LPS) stimulated retinal Angptl2 mRNA expression in vivo and in vitro. We generated EIU in wild-type (C57BL/6) and Angptl2−/− mice by injecting LPS intraperitoneally. Compared to wild-type animals, Angptl2−/− mice significantly reduced various EIU-associated cellular and molecular parameters including leukocyte adhesion to the retinal vessels and infiltration into the vitreous cavity and retinal mRNA expression levels of monocyte chemotactic protein-1, intercellular adhesion molecule-1, interleukin (IL)-6 and tumor necrosis factor (TNF)-α together with nuclear translocation of nuclear factor (NF)-κB p65 subunit. In vitro, antibody-based inhibition of α5β1 integrin, a receptor for Angptl2, significantly repressed LPS-induced expression of IL-6 and TNF-α, both of which are the major inflammatory cytokines derived from macrophages. The present findings indicate that Angptl2 mediates endotoxin-induced retinal inflammation through the activation of NF-κB signaling pathway and suggest a potential validity of Angptl2 as a new molecular target for the treatment of acute inflammation.
INTRODUCTION

Acute inflammation is the oldest defense mechanism in nature that is initiated by harmful irritation and environments such as infection and tissue injury.1, 2 Leukocytes in acute inflammation migrate to extravascular tissues to distinguish and eliminate the offending agent, and degrade necrotic tissue components, mostly contributing to tissue repair. In contrast to acute inflammation, leukocytes in chronic inflammation work damage on tissues because of continuous secretion of chemical mediators and toxic oxygen radicals, thereby developing in functional maladaptation and tissue remodeling.1, 2 However, even in acute inflammation, excessive and repeated acute attacks deteriorate into severe tissue damage and destruction, which is implicated in the pathogenesis of various diseases including vision-threatening uveo-retinal disorders such as Behçet’s disease.3

Injection of the bacterial endotoxin lipopolysaccharide (LPS) to animals induces acute systemic inflammation that is utilized as an established model for several inflammatory diseases including hepatitis,4 arthritis,5 and disseminated intravascular coagulation.6 LPS-induced ocular inflammation was originally considered as a model of acute inflammation of the uvea and thus termed endotoxin-induced uveitis (EIU). It is characterized by the breakdown of blood-ocular barrier represented by protein leakage into the anterior segment, leukocyte adhesion to the retinal vessels, and leukocyte infiltration into the vitreous cavity.7-10 These findings have expanded our understanding of the model as a tool to assess acute vascular inflammation not only in the uveal tract but also in the retina. EIU is regarded to represent acute intraocular inflammation as observed in Behçet’s disease.7-9 LPS application to vascular endothelial cells and macrophages stimulates the activation of nuclear factor (NF)-κB,11-14 a transcription factor that controls the expression of a variety of genes related to inflammatory responses, causing the development of diseases.11, 14, 15
Angiopoietin-like protein (Angptl) 2, which has the coiled-coil and fibrinogen-like domains that are conserved in angiopoietins, induces sprouting in human umbilical vein endothelial cells and is required for vascular development in Zebrafish.\textsuperscript{16,17} Recently, Angptl2 is reported to play a critical role in chronic inflammation of adipose tissue via inflammatory signaling in endothelial cells and subsequent recruitment of macrophages.\textsuperscript{18} Transgenic overexpression of Angptl2 in mouse skin caused chronic inflammation characterized by abundant attachment of leukocytes to the vessel walls and increased permeability, whereas Angptl2-deficient mice reduced inflammation and ameliorated systemic insulin resistance together with dietary obesity. Most recent studies have shown that Angptl2 also contributes to other chronic inflammatory disorders such as carcinogenesis and metastasis, dermatomyositis, and rheumatoid arthritis.\textsuperscript{19-22} Enhanced expression of Angptl2 has been observed in the sera or lesions of patients with obesity-related metabolic and cardiovascular diseases.\textsuperscript{18,23-25} These recent findings suggest robust evidence that Angptl2 mediates chronic inflammation. It remains unsolved, however, whether there is a relationship between Angptl2 and primitive acute inflammation. In the present study, we investigated the involvement of Angptl2 with endotoxin-induced acute inflammation in mice.
MATERIALS AND METHODS

Animals and Induction of EIU

C57BL/6 and Angptl2 knockout mice at the age of 8-10 weeks were maintained in the animal facility at Hokkaido University. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Ethics Review Committee for Animal Experimentation of Hokkaido University. Animals received a single intraperitoneal injection of 0.2 mg LPS from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS). Mice were evaluated at 3 (for NF-κB activation), 6 (for various gene expression) and 24 (for leukocyte infiltration) hours after LPS injection, all of which are the established time points for evaluation of pathological parameters in EIU.26-28

Cell Culture

Primary human retinal microvascular endothelial cells (HRMECs; Cell Systems, Kirkland, WA), hTERT-RPE, Y79 and RAW264.7 cells (American Type Culture Collection, Manassas, VA) were purchased. All cell lines were cultured following the manufacturers’ instructions. LPS was added at a final concentration of 30 ng/ml, and the cells were incubated for 6 hours. To inhibit of the bioactivity of α5β1 integrin, a receptor for Angptl2, RAW264.7 cells were incubated with rat anti-α5β1 integrin antibody (Millipore, Billerica, MA) or rat nonimmune control antibody (Millipore) for 30 minutes before LPS treatment.

Immunoblot Analysis

Protein extracts from mouse retinas were solubilized in 2x SDS (sodium dodecyl sulfate) sample buffer by heating to 100°C for 5 minutes and separated by 10% SDS-PAGE
(polyacrylamide gel electrophoresis). Proteins were transferred to PVDF (polyvinylidene fluoride) membrane by electroblotting, and immunoblot analyses were performed using goat anti-Angptl2 (R&D systems, Minneapolis, MN) and rabbit anti–ß actin antibodies (Cell Signaling Technology, Danvers, MA) as previously described.\textsuperscript{29}

**Immunofluorescence Microscopy**

Serial paraffin sections of mouse retinas were deparaffinized and hydrated through exposure with xylene and graded alcohols followed by water. As a pretreatment, microwave-based antigen retrieval was performed in 1 mM EDTA buffer (pH 8). Sections were probed with the following primary antibodies: goat anti-Angptl2, rabbit anti-NF-κB p65 (Cell Signaling Technology), and mouse anti-glutamine synthetase (Millipore) antibodies. The secondary antibodies for fluorescent detection were AlexaFluor 488 and 546 (Life Technologies, Carlsbad, CA). Sections were visualized under a FluoView FV10i-DOC (Olympus, Tokyo, Japan).

**Quantification of Retinal Adherent Leukocytes**

The retinal vasculature and adherent leukocytes were imaged by perfusion-labeling with fluorescein isothiocyanate (FITC)-coupled concanavalin A lectin (Con A; Vector, Burlingame, CA), as described previously.\textsuperscript{26-28} Briefly, the chest cavity was opened and a cannula was introduced into the left ventricle under deep anesthesia. After injection of PBS to remove erythrocytes and nonadherent leukocytes, FITC-conjugated Con A was perfused. After the eyes were enucleated, the retinas were flatmounted. The flatmounts were visualized under a Keyence BZ-9000 (Tokyo, Japan), and the total number of Con A-stained adherent leukocytes per retina was counted in a masked fashion.
Quantification of Vitreous Infiltrating Leukocytes

The number of leukocytes infiltrating into the vitreous cavity was analyzed as described previously. Briefly, tissues were fixed and embedded in paraffin using standard techniques. Three 5-µm sections were prepared at a distance of 100 µm to each other with the middle section passing through the optic nerve. All sections were stained with hematoxylin and eosin (H&E), and the number of cells in the vitreous cavity was counted in a masked fashion.

Real-Time Quantitative and Reverse Transcription PCR Analyses

Total RNA was isolated from tissues and cells using TRIzol (Life Technologies). Reverse transcription was performed with GoScrip Reverse Transcriptase (Promega, Madison, WI) and oligo dT(20) primers, essentially as described. Real-time quantitative PCR was performed using the GoTaq qPCR Master Mix (Promega), THUNDERBIRD Probe qPCR Mix (TOYOBO, Tokyo, Japan) and StepOne plus System (Life Technologies). All primers are listed in Table 1. Taqman gene expression assay for human HPRT1 (Life Technologies) was performed. The correct amplification of a specific product was confirmed by the dissociation temperature of the product and agarose gel electrophoresis. All experimental samples were normalized using human HPRT1 or mouse Gapdh as an internal control.

Enzyme Linked Immunosorbent Assay (ELISA) for NF-κB p65 Subunit

Nuclear extracts were purified from mouse retinas with Nuclear Extraction Kit (Active Motif, Carlsbad, CA). Activation of NF-κB was determined by measuring the NF-κB subunit p65 protein level in the nuclear extracts with an NF-κB detection kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instruction. The tissue sample concentration
was calculated from a standard curve and corrected for protein concentration.

**Statistical Analysis**

All results were expressed as mean ± SEM (standard error for mean). The values were processed for statistical analysis (Student’s t-test). Differences between the means were considered statistically significant when the $P$ values were $< 0.05$. 
RESULTS

Angptl2 Expression in Retinas of Wild-Type but Not Angptl2<sup>−/−</sup> Mice

Angptl2 mRNA has recently proven to be expressed in the heart, lung, kidney, skeletal muscle, and adipose tissue; however, retinal expression remains to be clarified. Immunoblot analysis revealed a predicted protein band of approximately 57 kDa in wild-type mouse retina (Figure 1a), and Angptl2 immunoreactivity were widely distributed in the retina (Figure 1b, c), whereas both immunoblot and immunofluorescence signals were abolished in Angptl2<sup>−/−</sup> mice (Figure 1a, d, e). We performed histological and real-time quantitative PCR analyses to check whether Angptl2 deficiency affects retinal development. Angptl2<sup>−/−</sup> mice exhibited no obvious morphological phenotype in the retina at 8 weeks of age (Figure 1f, g). No significant difference was detected in gene expression of various retinal cell markers including Rho (rhodopsin), Opn1mw [opsin 1 (cone pigments), medium-wave-sensitive], Grm6 (glutamate receptor, metabotropic 6) and Thy1 (thymus cell antigen 1) between wild-type and Angptl2<sup>−/−</sup> mice (Rho, fold change = 1.01; Opn1mw, fold change = 1.06; Grm6, fold change = 1.11; Thy1, fold change = 1.07; P > 0.05 for all) (Figure 1h).

LPS-Stimulated Induction of Angptl2 mRNA Expression

To define the association of Angptl2 with acute inflammation, we examined Angptl2 gene expression following the in vivo and in vitro stimulation with LPS by real-time quantitative PCR. Angptl2 mRNA level was significantly increased in the retina of EIU mice compared to that of normal controls (fold change = 3.42, P < 0.01) (Figure 2a). In vitro, LPS administration led to significant upregulation of ANGPTL2 (Angptl2 for mice) gene expression in various cell lines including HRMEC (retinal microvasculcular endothelial cells), hTERT-RPE (retinal pigment epithelial cells), Y79 (retinoblastoma cells) and RAW264.7
Reduction of Retinal Leukocyte Adhesion in \( \text{Angptl2}^{-/-} \) Mice with EIU

To examine whether \( \text{Angptl2} \) deficiency alters acute retinal inflammation, we evaluated the number of leukocytes adhering to the retinal vessels in wild-type, \( \text{Angptl2}^{+/+} \) and \( \text{Angptl2}^{-/-} \) mice with EIU. Compared with wild-type animals with EIU (232 ± 26 cells/retina; Figure 3a, b, g), EIU \( \text{Angptl2}^{-/-} \) mice showed a significant (\( P < 0.01 \)) decrease in retinal leukocyte adhesion to 49.1% (114 ± 24 cells/retina; Figure 3e, f, g), whereas no significant difference was detected between wild-type and \( \text{Angptl2}^{+/+} \) mice with EIU (232 ± 26 vs. 200 ± 16 cells/retina, \( P > 0.05 \); Figure 3c, d, g).

Reduction of Vitreous Leukocyte Infiltration in \( \text{Angptl2}^{-/-} \) Mice with EIU

To further confirm the inhibitory effect of \( \text{Angptl2} \) deficiency on acute retinal inflammation (Figure 3), we quantified the number of leukocytes infiltrating into the vitreous cavity adjacent to the optic disc in wild-type, \( \text{Angptl2}^{+/+} \) and \( \text{Angptl2}^{-/-} \) mice with EIU. Leukocyte infiltration into the vitreous, which markedly increased with induction of EIU, significantly decreased in \( \text{Angptl2}^{-/-} \) mice to 22.9% (wild-type, 35 ± 8 cells/section; \( \text{Angptl2}^{+/+} \), 25 ± 9 cells/section, \( P > 0.05 \); \( \text{Angptl2}^{-/-} \), 8 ± 3 cells/section, \( P < 0.01 \)) (Figure 4a-d).

Reduction of Retinal Inflammatory Molecules in \( \text{Angptl2}^{-/-} \) Mice with or without EIU
To determine molecular mechanisms in which Angptl2 deficiency led to suppression of cellular inflammatory responses in EIU (Figures 3 and 4), retinal mRNA expression levels of C-C chemokine ligand (CCL) 2/monocyte chemotactic protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured by real-time quantitative PCR. Compared with wild-type normal controls, Angptl2 deficiency decreased the endogenous baseline expression of all these inflammation-related molecules (CCL2/MCP-1, fold change = 0.406, $P < 0.01$; ICAM-1, fold change = 0.445, $P < 0.05$; IL-6, fold change = 0.170, $P < 0.01$; TNF-α, fold change = 0.473, $P < 0.05$) (Figure 5a). Subsequently, we measured the gene expression levels in EIU. EIU induction to wild-type mice dramatically upregulated all of the four genes compared with normal controls (CCL2/MCP-1, fold change = 1136.8, $P < 0.01$; ICAM-1, fold change = 54.15, $P < 0.05$; IL-6, fold change = 3.10, $P < 0.01$; TNF-α, fold change = 1.53, $P < 0.05$), as reported previously.\textsuperscript{26-28} Compared with wild-type EIU, Angptl2 deficiency also declined the EIU-associated upregulated expression of all these inflammatory molecules (CCL2/MCP-1, fold change = 0.318, $P < 0.01$; ICAM-1, fold change = 0.246, $P < 0.01$; IL-6, fold change = 0.545, $P < 0.05$; TNF-α, fold change = 0.659, $P < 0.05$) (Figure 5b).

**Reduction of NF-κB Activation in Angptl2\textsuperscript{−/−} Mice with EIU**

To determine the upstream intracellular signaling pathway of the reduced cellular (Figures 3 and 4) and molecular (Figure 5) inflammatory responses in Angptl2\textsuperscript{−/−} EIU, we examined the nuclear translocation of NF-κB p65 by immunofluorescence. EIU induction to wild-type mice substantially increased the number of NF-κB p65-positive cells colocalized with immunoreactivity of glutamine synthetase, suggesting the activation of NF-κB p65 in glutamine synthetase-positive Müller glial cells (Figure 6a, b). Compared with wild-type
EIU, Angptl2 deficiency led to significant ($P < 0.05$) suppression of the EIU-associated nuclear translocation of NF-κB p65 by 38.9% (Figure 6c-e).

To confirm the morphometric data (Figure 6c-e), we quantitatively measured nuclear protein levels of NF-κB p65 by ELISA following nuclear extraction from the whole retina. Compared with wild-type EIU, Angptl2 deficiency led to significant ($P < 0.05$) reduction of the EIU-associated nuclear NF-κB p65 protein levels by 17.5% (Figure 6f).

**Reduction of LPS-Stimulated Cytokine Expression by Inhibiting α5β1 Integrin**

Angptl2 interacts with α5β1 integrin that activates its downstream NF-κB pathway in vascular endothelial cells and macrophages.\textsuperscript{18, 22} To investigate whether LPS-stimulated Angptl2 induces inflammatory responses via α5β1 integrin, we performed in vitro experiments with a neutralizing antibody. First, we confirmed using reverse transcription PCR analyses the constitutive expression of Itga5 (integrin α5 subunit) and Igtb1 (integrin β1 subunit) in RAW264.7 macrophages (Figure 7a), which proved to be the rich source of Angptl2 (Figure 2e). LPS administration to macrophages significantly upregulated the two major inflammatory cytokines (TNF-α, fold change = 34.7, $P < 0.01$; IL-6, fold change = 7947.4, $P < 0.01$), both of which were significantly suppressed by anti-α5β1 antibody (TNF-α, fold change = 22.0, $P < 0.01$; IL-6, fold change = 4728.8, $P < 0.05$), but not by control antibody (TNF-α, fold change = 33.7, $P > 0.05$; IL-6, fold change = 7836.6, $P > 0.05$) (Figure 7b, c). LPS application to RAW264.7 cells did not alter Itga5 or Igtb1 gene expression (data not shown), suggesting a ligand-based regulation in the Angptl2/α5β1-mediated proinflammatory system.
DISCUSSION

The present study provided, for the first time to our knowledge, several important data concerning the biological effects of Angptl2 on acute retinal inflammation. First, Angptl2 was shown to be ubiquitously expressed in the retina (Figure 1). Second, LPS stimulation in vitro and in vivo significantly induced Angptl2 mRNA expression (Figure 2). Importantly, Angptl2 deficiency in EIU attenuated several cellular and molecular inflammatory parameters including leukocyte adhesion (Figure 3) and infiltration (Figure 4), the upregulated expression of various inflammatory mediators (Figure 5), and NF-κB activation (Figure 6). Moreover, LPS-induced expression of inflammatory cytokines in vitro was significantly suppressed by inhibiting α5β1 integrin, a receptor for Angptl2 (Figure 7).

The present study is the first to show the constitutive expression of Angptl2 in the retina, whereas Angptl2 mRNA has so far been detected mainly in the heart, lung, kidney, skeletal muscle, and adipose tissue, most of which are the target organs for lifestyle-related metabolic disorders. Importantly, Angptl2−/− mice did not show any remarkable changes in retinal morphology and gene expression of various retinal cell markers (Figure 1). These results suggest that Angptl2 is not required for normal retinal development although its broad distribution in the whole retinal layer suggests some possible role in adults.

Recently, Angptl2 mRNA expression has proven to be enhanced by hypoxia and palmitate-induced endoplasmic reticulum (ER) stress in adipocytes, indicating its possible role in obesity-related chronic inflammation. Our data first revealed that Angptl2 mRNA expression was stimulated by the bacterial endotoxin LPS (Figure 2), a known inducer of primitive acute inflammation. We confirmed that the endotoxin-induced Angptl2 mRNA expression was observed in the retina as well as in multiple retinal cell lines and macrophages related to the pathogenesis of EIU (Figure 2). Palmitate application to pancreatic β-cells has been reported to activate the ER stress-induced transcription factor CHOP (C/EBP
Interestingly, LPS stimulation to macrophages also induced the ER stress-CHOP pathway. However, our in vitro experiments with small interfering RNA-based knockdown of CHOP revealed that this transcription factor did not mediate LPS-stimulated expression of Angptl2 in macrophages (data not shown), suggesting different induction pathways for Angptl2 between bacterial endotoxin and saturated fatty acid, which cause acute and chronic inflammation, respectively.

EIU is a useful model for assessing acute vascular inflammation involving the major components in the innate immune system. \(^7\) \(^\text{10}\) Angptl2 \(^{-/-}\) mice with EIU exhibited significant reduction of accumulation of adherent leukocytes to the retinal vessels (Figure 3) and infiltrating leukocytes into the vitreous cavity (Figure 4). These findings provide the first evidence that Angptl2 plays a significant role in acute inflammation. In addition to the investigation into cellular reactions, comparable experiments clarified significant suppression of CCL2/MCP-1 and ICAM-1 mRNA levels in Angptl2 \(^{-/-}\) mice (Figure 5), both of which play pivotal roles in leukocyte recruitment and adhesion during vascular inflammation. \(^33\) \(^34\) The interaction between ICAM-1 and its counter receptor β2 (CD18)-integrins [i.e., LFA (leukocyte function-associated antigen)-1 and Mac-1] has been shown to play an essential role in the leukocyte-endothelial interaction in the pathogenesis of EIU. \(^34\) \(^35\) ICAM-1 expression is upregulated in the retina following LPS application, and antibody-based blockade of ICAM-1 leads to significant suppression of EIU development. \(^34\) CCL2/MCP-1, the major chemotactic activator of monocytes and neutrophils, is required for leukocyte recruitment to the sites of inflammation in EIU, \(^36\) since genetic ablation of CCL2/MCP-1 leads to a substantial decrease in ocular inflammation in mice with EIU. \(^37\) Moreover, we unraveled the inhibitory effects of Angptl2 deficiency on gene expression levels of the two major pro-inflammatory cytokines IL-6 and TNF-α (Figure 5), both of which facilitate ocular inflammation in EIU, \(^9\) \(^38\) in concert with the leukocyte chemotactic and adhesion factors
CCL2/MCP-1 and ICAM-1. Consequently, Angptl2 deficiency-mediated attenuation of these inflammatory chemical mediators (Figure 5) is thought to result in decreased vascular inflammation in EIU (Figures 3 and 4).

The present study revealed that Angptl2 deficiency led to significant suppression of EIU-associated nuclear translocation of NF-κB (Figure 6). NF-κB, the p65/p50 heterodimer, is a transcription factor that regulates numerous gene expression coordinating inflammatory responses. Activation of NF-κB signaling contributes to the development of various inflammatory disorders such as EIU,11-15, 39 activating gene expression including CCL2/MCP-1, ICAM-1, IL-6 and TNF-α,14, 15, 40, 41 all of which were suggested to enhance acute inflammation in the downstream of Angptl2 (Figure 5). Angptl2 has recently been shown to interact with cell-surface α5β1 integrin that mediates its downstream NF-κB activation in vascular endothelial cells and macrophages.18, 22 Furthermore, administration of recombinant Angptl2 protein to keratinocytes enhanced the expression of IL-1β and IL-6 via the NF-κB cascade.22 These recent findings are compatible with our present data suggesting that LPS-induced retinal expression of Angptl2 (Figures 1 and 2) causes NF-κB activation (Figure 6) and subsequent molecular (Figure 5) and cellular (Figures 3 and 4) reactions. Moreover, LPS-induced expression of TNF-α and IL-6 in Angptl2-bearing macrophages (Figure 2e) was mediated by α5β1 integrin (Figure 7). The in vitro data further confirmed the Angptl2/α5β1-mediated proinflammatory signaling in macrophages, the major leukocytes infiltrating into the eye with EIU. Accordingly, these infiltrating cells, as well as the inflamed retina, would also be the source of Angptl2 (Figure 2a), phosphorylated NF-κB (Figure 6f) and inflammatory cytokines TNF-α and IL-6 (Figure 5b), forming a vicious cycle to amplify acute inflammation.

In conclusion, Angptl2 is likely to play a significant role in inducing acute inflammation, sharing at least in part its downstream molecular signaling as involved in
chronic inflammation. The current results suggest a potential validity of Angptl2 as a therapeutic target for various inflammatory diseases.
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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest concerning this work.
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FIGURE LEGENDS

Figure 1. Angptl2 Expression in Retinas of Wild-Type but Not Angptl2+/− Mice.

(a) Immunoblotting for Angptl2 protein. Protein extracts were prepared from retinas of wild-type and Angptl2+/− mice. (b-e) Localization of Angptl2 protein in mouse retinas. Angptl2 (green) and DAPI (blue). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 30 µm. (f, g) H&E staining of wild-type and Angptl2+/− mice retina at 8 weeks of age. (h) Relative mRNA expression in wild-type and Angptl2+/− mice retinas. Fold changes in expression of target genes (Rho, Opn1mw, Grm6 and Thy1) relative to the internal control. Wild-type gene expression was used as a reference (n = 7 per group). White and black bars indicate wild-type and Angptl2+/− mice, respectively.

Figure 2. LPS-Stimulated Induction of Angptl2 mRNA Expression.

(a) Angptl2 mRNA expression in wild-type mice retina with EIU. Angptl2 mRNA expression of LPS-treated HRMEC (b), hTERT-RPE (c), Y79 (d) and RAW264.7 cells (e). Real-time quantitative PCR for Angptl2 after stimulation with LPS. *P < 0.05, **P < 0.01 (n = 6 per group).

Figure 3. Reduction of Retinal Leukocyte Adhesion in Angptl2+/− Mice with EIU.

Flatmounted retinas from wild-type (a, b), Angptl2+/− (c, d) and Angptl2+/− mice (e, f) with EIU. Arrows indicate firmly adhering leukocytes to the inflamed retinal vasculature. Scale bar, 100 µm. (g) Quantification of the number of retinal adherent leukocytes. **P < 0.01 (n = 10 to 11 per group). White, gray and black bars indicate wild-type, Angptl2+/− and Angptl2+/− mice, respectively.
Figure 4. Reduction of Vitreous Leukocyte Infiltration in Angptl2-/- Mice with EIU.

Representative micrographs of leukocyte infiltration into the vitreous of wild-type (a), Angptl2+/+ (b) and Angptl2-/- mice (c) with EIU. Arrows indicate the infiltrating leukocytes. Scale bar, 30 µm. (d) Quantification of the number of leukocytes in the vitreous cavity. **P < 0.01 (n = 8 per group). White, gray and black bars indicate wild-type, Angptl2+/+ and Angptl2-/- mice, respectively.

Figure 5. Reduction of Retinal Inflammatory Molecules in Angptl2-/- Mice with or without EIU.

(a) Gene expression levels of CCL2/MCP-1, ICAM-1, IL-6 and TNF-α in retinas from wild-type and Angptl2-/- mice. (b) Gene expression levels of CCL2/MCP-1, ICAM-1, IL-6 and TNF-α in retinas from wild-type and Angptl2-/- mice with EIU. *P < 0.05, **P < 0.01 (n = 7 to 9 per group). Wild-type gene expression was used as a reference. White and black bars indicate wild-type and Angptl2-/- mice, respectively.

Figure 6. Reduction of NF-κB Activation in Angptl2-/- Mice with EIU.

Immunofluorescence for NF-κB p65 (green) and glutamine synthetase (GS) (red) in the retinal sections from wild-type mice without (a) and with (b) EIU. Localization of NF-κB p65 in retinas from wild-type (c) and Angptl2-/- (d) mice with EIU. NF-κB p65 (green) and DAPI (blue). INL, inner nuclear layer. Scale bar, 10 µm. Arrows indicate merged images of NF-κB p65 and DAPI staining. (e) The number of nuclear NF-κB p65-positive cells in the inner nuclear layer. (f) Nuclear NF-κB p65 protein levels detected by ELISA after nuclear extraction from the whole retina. *P < 0.05, **P < 0.01 (n = 9 to 13 per group). White and black bars indicate wild-type and Angptl2-/- mice with EIU, respectively.
Figure 7. Reduction of LPS-Stimulated Cytokine Expression by Inhibiting α5β1 Integrin

(a) Gene expression analysis of *Itga5* and *Itgb1* in RAW264.7 cells. Real-time quantitative PCR for *TNF-α* (b) and *IL-6* (c) in RAW264.7 cells. RAW264.7 cells were pre-incubated with anti-α5β1 integrin or control antibody before stimulation with LPS. *P < 0.05, **P < 0.01 (n = 8 per group).
Figure 1

(a) SDS-PAGE gel showing the expression levels of Wild-type and Angptl2^−/− proteins. IB: Anti-Angptl2 and IB: Anti-β actin.

(b-d) Immunoblot images of Wild-type (b) and Angptl2^−/− (d) showing the expression of Angptl2.

(c-e) Immunofluorescence images of Wild-type (c) and Angptl2^−/− (e) showing the expression of ONL, INL, and GCL.

(h) Relative mRNA expression (fold change) comparison between Wild-type and Angptl2^−/− for various genes: Rip, Gm3, Gm5, and Thy1.
Figure 2

(a) Mouse retina

(b) HRMEC

(c) hTERT-RPE

(d) Y79

(e) RAW264.7
Figure 3

Leucocyte counts (cells/retina)

Wild-type
Angptl2−/−
Angptl2+/−

Posterior retina
Peripheral retina

Leucocyte counts (cells/retina)

Wild-type
Angptl2−/−
Angptl2+/−

**
Figure 4

(a) Wild-type

(b) Angptl2++

(c) Angptl2-/

(d) Leukocyte counts (cells/section)

** Wild-type Angptl2++ Angptl2-/

0 20 40 60
Figure 5

(a) Relative mRNA expression (fold change) for CCL2/MCP-1, ICAM-1, IL-6, and TNF-α in Wild-type and Angptl2-/- mice. Significant differences are indicated by asterisks: **p < 0.01, *p < 0.05.

(b) EIU experiment showing the relative mRNA expression (fold change) for CCL2/MCP-1, ICAM-1, IL-6, and TNF-α in Wild-type and Angptl2-/- mice. Significant differences are indicated by asterisks: **p < 0.01, *p < 0.05.
Figure 6

(a) Normal Wild-type
(b) EIU Wild-type
(c) EIU Wild-type
(d) EIU Angptl2^{-/-}
(e) Nuclear NF-κB p65 positive cells in inner nuclear layer (% of wild-type)
(f) Nuclear NF-κB p65 protein levels (total retinal nuclear extract ratio)
Figure 7

a

DNA marker  Itga5  Ilgb1  Gapdh  H2O

b

Relative TNF-α mRNA expression (fold change)

N.S.

**

LPS  -  +  +  +
Anti-α5β1 Ab  -  -  +  -
Control Ab  -  -  -  +

C

Relative IL-6 mRNA expression (fold change)

N.S.

**  *

LPS  -  +  +  +
Anti-α5β1 Ab  -  -  +  -
Control Ab  -  -  -  +