Interleukin-6 Receptor-Mediated Activation of Signal Transducer and Activator of Transcription-3 (STAT3) Promotes Choroidal Neovascularization

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Interleukin (IL)-6, a potent proinflammatory cytokine, is suggested to be a risk factor for choroidal neovascularization (CNV) because of its increased levels in the serum of patients with age-related macular degeneration; however, the role of IL-6 in CNV has not been defined. The present study reveals the critical contribution of IL-6 signaling and its downstream STAT3 pathway to the murine model of laser-induced CNV. CNV induction by laser treatment stimulated IL-6 expression in the retinal pigment epithelium-choroid complex, and antibody-based blockade of IL-6 receptor or genetic ablation of IL-6 led to significant suppression of CNV. CNV generation was accompanied by STAT3 activation in choroidal endothelial cells and macrophages, and IL-6 receptor blockade resulted in selectively inhibited phosphorylation of STAT3 but not extracellular signal-regulated kinase 1/2. Consistently, pharmacological blockade of STAT3 pathway also suppressed CNV. In addition, IL-6 receptor neutralization led to significant inhibition of the *in vivo* and *in vitro* expression of inflammation-related molecules including monocyte chemotactic protein, intercellular adhesion molecule-1, and vascular endothelial growth factor, and of macrophage infiltration into CNV. These results indicate the significant involvement of IL-6 receptor-mediated activation of STAT3 inflammatory pathway in CNV generation, suggesting the possibility of IL-6 receptor blockade as a therapeutic strategy to suppress CNV associated with age-related macular

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Age-related macular degeneration (AMD) is the most common cause of blindness in developed countries.¹ The macula is located at the center of the retina, and the visual acuity depends on the function of the macula where cone photoreceptors are abundant. AMD is complicated by choroidal neovascularization (CNV), leading to severe vision loss and blindness. During CNV generation, new vessels from the choroid invade the subretinal space through Bruch's membrane, resulting in the formation of the neovascular tissue including vascular endothelial cells, retinal pigment epithelial cells, fibroblasts, and macrophages.² Subretinal hemorrhage and lipid exudation develop from the immature vessels in the proliferative tissue, causing injury to the photoreceptors.

Molecular and cellular mechanisms underlying CNV are not fully elucidated. CNV seen in AMD develops with chronic inflammation adjacent to the retinal pigment epithelium (RPE), Bruch's membrane, and choriocapillaris. Recent experimental and clinical studies have indicated vascular endothelial growth factor (VEGF) as a critical factor for promoting CNV.^{3,4} CNV formation is associated with the influx of inflammatory cells including macrophages, which are shown to be a rich source of VEGF. Pharmacological depletion of macrophages, present in both human and murine CNV tissues,^{2,5–7} results in significant suppression of murine CNV.^{5,7} CNV tissues from both human surgical samples and the rodent laser-induced model express inflammation-related molecules including intercellular adhesion molecule (ICAM)-1.^{8,9} Genetic ablation of ICAM-1 or CC chemokine receptor-2, a

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receptor for monocyte chemotactic protein (MCP)-1, inhibited CNV in the murine model.^{6,9}

Interleukin (IL)-6 is a potent proinflammatory cytokine that binds to its receptor IL-6R, and the complex of IL-6 and IL-6R interacts with gp130 on the cell surface, leading to dimerization of gp130 that initiates IL-6-mediated signaling in target cells.^{10,11} Because of the soluble, diffusible form of IL-6R in addition to membrane-bound IL-6R, the complex of IL-6 and soluble IL-6R is capable of inducing IL-6-mediated signal transduction even in IL-6R-negative cells, if only they express gp130.¹¹ Downstream pathways following gp130 dimerization include the activation of STAT3 (signal transducer and activator of transcription 3), a known transcription factor that induces inflammation,^{12,13} and ERK (extracellular signal-regulated kinase) MAP (mitogen-activated protein) kinase cascade, which mainly promotes cell proliferation.^{14,15}

Recently, IL-6 has been suggested to play a role in the pathogenesis of ocular diseases. Vitreous aspirates from patients with proliferative diabetic retinopathy, another vision-threatening disease characterized by retinal neovascularization, exhibit the parallel increases in IL-6 and VEGF.¹⁶ Interestingly, increased serum levels of IL-6 and C-reactive protein have recently proven to be related with progression of AMD.¹⁷ No data have been reported, however, showing the direct evidence of the pathogenic role of IL-6 signaling in CNV generation. Here, we report the first evidence of the *in vivo* effect of IL-6R blockade on the murine model of CNV, together with underlying molecular and cellular mechanisms.

Materials and Methods

Animals

Male C57BL/6J mice (CLEA, Tokyo, Japan) at the age of 7 to 10 weeks and age- and sex-matched IL-6-deficient homozygous mice raised on C57BL/6J background (Jackson Laboratories, Bar Harbor, ME) were used. All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of CNV

Laser-induced CNV is widely used as an animal model for neovascular AMD and reflects the pathogenesis of choroidal inflammation and neovascularization seen in AMD. In this model, new vessels from the choroid invade the subretinal space after photocoagulation. Laser photocoagulation was performed at five spots per eye around the optic disk with the wavelength of 532 nm, the power of 200 mW, the duration of 100 ms, and the spot size of 75 μ m, using a slit-lamp delivery system (Novus Spectra; Lumenis, Tokyo, Japan), as described previously.⁵

Treatment with an IL-6R Neutralizing Antibody or a JAK2 Tyrosine Kinase Inhibitor

Animals were treated with an intraperitoneal injection of a rat anti-mouse IL-6R monoclonal antibody MR16-1 (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan), prepared as described previously,¹⁸ or a purified rat nonimmune isotype IgG (MP Biomedicals, Solon, OH) immediately after photocoagulation. MR16-1 has been shown to bind to murine IL-6R and suppress IL-6-induced cellular responses in a dose-dependent manner.¹⁹ Other basic properties of this antibody have been described in previously published reports.^{18,19} MR16-1 was injected into mice with the dose of 1, 10, or 100 μ g/g body weight. Animals were also treated with a JAK2 tyrosine kinase inhibitor AG490 (Calbiochem, La Jolla, CA) or phosphate-buffered saline (PBS) containing 1% dimethyl sulfoxide as vehicle daily for 3 days after photocoagulation. AG490 was shown to inhibit JAK/STAT pathway and suppress the growth of various cancers.^{20,21} AG490 was intraperitoneally administered to mice with the dose of 0.1 or 1 μ g/g body weight.

Quantification of Laser-Induced CNV

One week after laser injury, eyes were enucleated and fixed with 4% paraformaldehyde. Eye cups obtained by removing anterior segments were incubated with 0.5% fluorescein isothiocyanate-isolectin B4 (Vector, Burlingame, CA). CNV was visualized with blue argon laser wavelength (488 nm) using a scanning laser confocal microscope (FV1000; Olympus, Tokyo, Japan). Horizontal optical sections of CNV were obtained every 1- μ m step from the surface to the deepest focal plane. The area of CNV-related fluorescence was measured by National Institutes of Health Image (Bethesda, MD). The summation of whole fluorescent area was used as the index of CNV volume, as described previously.⁵

Immunohistochemistry for Phosphorylated STAT3

Immunohistochemical experiments were performed for murine CNV. Murine eyes enucleated 72 hours after photocoagulation were fixed with acetone at 4°C and embedded in paraffin. After blocking nonspecific binding in PBS containing 1% bovine serum albumin for 30 minutes at room temperature, paraffin sections were incubated overnight at 4°C with a rabbit anti-phosphorylated STAT3 antibody (1:100; Cell Signaling Technology, Beverly, MA) together with a rat polyclonal antibody against F4/80 (1:100; Serotec, Oxford, UK) or 0.5% fluorescein isothiocyanate-isolectin B4. Avidin-Alexa 488- and avidin-Alexa 546-tagged secondary antibodies (1:200; Molecular Probes, Eugene, OR) were then applied for 2 hours at room temperature. For nuclear staining, the specimens were treated with TOTO-3 (1:500; Molecular Probes) at room temperature for 30 minutes. After two washes, the samples were viewed with the scanning laser confocal microscope.

Western Blot Analyses

Protein extracts were obtained from the homogenized RPE-choroid complex 1 day after photocoagulation. The choroid was carefully isolated and placed into 100 μ L of lysis buffer (0.02 mol/L HEPES, 10% glycerol, 10 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 100 μ mol/L Na_3VO_4 , 1% Triton X-100, 100 mmol/L NaF, and 4 mmol/L ethylenediaminetetraacetic acid, pH 8.0) supplemented with protease inhibitors (2 mg/L aprotinin, 100 μ mol/L phenylmethyl sulfonyl fluoride, 10 μ mol/L leupeptin, and 2.5 μ mol/L pepstatin A) and sonicated. The lysate was centrifuged, and the supernatant was collected. Each sample containing 30 μ g of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to polyvinylidene fluoride membrane (ATTO, Tokyo, Japan). After blocking nonspecific binding with 5% skim milk, the membranes were incubated with a rabbit polyclonal antibody against STAT3 (1:1000), ERK1/2 (1:2000), phosphorylated forms of STAT3 or ERK1/2 (1:1000; Cell Signaling Technology), or an anti- α -tubulin antibody (1:2000; Sigma, St. Louis, MO) at 4°C overnight, followed by incubation with a horseradish peroxidase-conjugated goat antibody against rabbit IgG (1:5000; BioSource, Camarillo, CA). The signals were visualized with an enhanced chemiluminescence kit (GE Health Care, Buckinghamshire, UK) according to the manufacturer's protocol.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analyses for Various Inflammation-Related Molecules

Total RNA was isolated from the RPE-choroid complex 3 days after photocoagulation using an extraction reagent (TRIzol; Invitrogen, Carlsbad, CA) and reverse-transcribed with a cDNA synthesis kit (First-Strand; GE Health Care). PCR was performed using *Taq* DNA polymerase (Takara Bio, Ohtu, Japan) in a thermal controller (Gene Amp PCR system; Applied Biosystems, Foster, CA). The primer sequences and the expected size of amplified cDNA fragments are as follows: 5'-TTCCTCTCTGCAAGAGACT-3' (sense) and 5'-TGTATCTCTCTGAGGACT-3' (anti-sense) (430 bp) for *IL-6*, 5'-GTGTCGAGCTTTGGGATGGTA-3' (sense) and 5'-CTGGGCTTGAGACTCAGTG-3' (anti-sense) (505 bp) for *ICAM-1*, 5'-CCCCACTCACCTGCTGCTACT-3' (sense) and 5'-GGCATCACAGTCCGAGTCACA-3' (anti-sense) (380 bp) for *MCP-1*, 5'-GAAGTCCCATGAAGTGATCCAG-3' (sense) and 5'-TCACCGCCTTGGCTTGTC-3' (anti-sense) (319 bp and 451 bp) for *VEGF120* and *VEGF164*, respectively, and 5'-ATGTGGCACCACACCTTCTACAATGAGCTGCG-3' (sense) and 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3' (anti-sense) (837 bp) for β -actin.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-6, ICAM-1, MCP-1, and VEGF

The RPE-choroid complex was carefully isolated from the eyes 3 days after photocoagulation and placed into 100 μ L

of lysis buffer supplemented with protease inhibitors and sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C, and the levels of IL-6, ICAM-1, MCP-1, and VEGF were determined with the mouse IL-6, ICAM-1, MCP-1, and VEGF ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols.

Quantification of Infiltrating Macrophages

Three days after laser injury, eyes were enucleated and whole-mount choroid-sclera complexes were incubated overnight at 4°C with a goat polyclonal antibody against mouse PECAM-1 (CD31) and a rat polyclonal antibody against F4/80 (Serotec). Avidin-Alexa 488- and avidin-Alexa 546-tagged secondary antibodies were then applied for 2 hours at room temperature, and CNV was viewed with the scanning laser confocal microscope. PECAM-1-stained area of CNV was measured as fluorescent pixels, the number of F4/80-positive macrophages was counted in every 5- μ m step of CNV, and area-adjusted number of macrophages per 10,000- μm^2 area of CNV was calculated.

In Vitro Assays

Murine brain-derived capillary endothelial cell line (b-End3) and murine macrophages (RAW264.7) were cultured with Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum at 37°C in a 95% air-5% CO_2 atmosphere. Lipopolysaccharide (LPS; Sigma) was used as a potent inducer of IL-6. Twelve hours before the experiments with b-End3 cells, the culture medium was changed to serum-free Dulbecco's modified Eagle's medium. After a 2-hour incubation with LPS (200 ng/ml) plus MR16-1 (1 or 10 μ g/ml) or LPS plus control rat IgG (10 μ g/ml), the cell lysate was processed for Western blot analyses for total and phosphorylated STAT3, and total cellular RNA was processed for RT-PCR analyses for *ICAM-1* and *MCP-1*. For protein analyses, the supernatant and cell lysate were collected after a 6-hour incubation and then the concentration of MCP-1 in the supernatant and ICAM-1 in the cell lysate were measured by the ELISA kits (R&D Systems).

RAW264.7 cells were treated with Dulbecco's modified Eagle's medium containing LPS (200 ng/ml) plus MR16-1 (1 or 10 μ g/ml) or LPS plus control rat IgG (10 μ g/ml). After a 2-hour incubation, the cell lysate was collected for Western blot analyses for STAT3. After a 6-hour incubation, the supernatant and total cellular RNA were processed for ELISA and RT-PCR analyses for VEGF.

Morphometric and Statistical Analyses

All results were expressed as mean $\pm$ SD. The values were processed for statistical analyses (Mann-Whitney test). Differences were considered statistically significant when $P < 0.05$.

Figure 1. Up-regulation of IL-6 in the RPE-choroid complex by inducing CNV. RT-PCR (**A**) and ELISA (**B**) analyses for expression and production of IL-6 in the RPE-choroid complex. $n = 8$ to 9 . * $P < 0.01$.

Results

Up-Regulation of IL-6 by Inducing CNV

The RPE-choroid complex was subjected to RT-PCR and Western blot analyses to detect the expression of IL-6 mRNA (Figure 1A) and protein (Figure 1B), respectively. mRNA expression of *IL-6* was higher in the RPE-choroid complex of mice 1 and 3 days after photocoagulation than in age-matched normal controls (Figure 1A). mRNA expression of *IL-6* in the RPE-choroid complex was returned to the normal level 7 and 10 days after photoco-

agulation. Similarly, IL-6 protein levels at 3 days after photocoagulation were significantly increased by inducing CNV ($P < 0.01$) (Figure 1B).

Suppression of CNV in Mice Receiving IL-6R-Neutralizing Antibody or Deficient in IL-6

The index of CNV volume was measured to evaluate the effects of the IL-6R signaling on the development of CNV. CNV was significantly suppressed by blocking IL-6R signaling with MR16-1. MR16-1-treated mice at the dose of 10 or 100 $\mu\text{g/g}$ showed a significant ($P < 0.001$) decrease in the index of CNV volume ($312,076 \pm 87,973 \mu\text{m}^3$ for 10 $\mu\text{g/g}$, $349,720 \pm 104,395 \mu\text{m}^3$ for 100 $\mu\text{g/g}$) compared with control IgG-treated mice ($508,423 \pm 136,303 \mu\text{m}^3$) (Figure 2, A and B). In addition, CNV was also significantly ($P < 0.001$) attenuated in IL-6-deficient mice ($359,878 \pm 110,767 \mu\text{m}^3$) to the similar levels of IL-6R blockade, compared with wild-type animals ($510,630 \pm 153,019 \mu\text{m}^3$) (Figure 2, C and D).

Figure 2. Suppression of CNV in mice receiving anti-IL-6R antibody or deficient in IL-6. **A** and **C**: The graphs show the index of CNV volume. **B** and **D**: Flat-mounted choroids from control IgG-treated mice, MR16-1-treated mice (1, 10, and 100 $\mu\text{g/g}$ body weight), wild-type animals, and IL-6 knockouts (KO). Arrowheads indicate lectin-stained CNV tissues. $n = 6$ to 30 . ** $P < 0.001$. Scale bars = 50 μm .

Figure 3. Tissue localization of phosphorylated STAT3 in murine CNV. The immunohistochemical analyses showed phosphorylated STAT3 staining in isolectin B4-positive endothelial cells (**A**) and F4/80-positive macrophages (**B**). **A:** Green fluorescence from isolectin B4 (left) and red fluorescence from an anti-phosphorylated STAT3 antibody (middle) identified the isolectin B4-positive endothelial cells as expressing phosphorylated STAT3 when the images were superimposed (**arrowheads** at right). **B:** Similarly, green fluorescence from an anti-F4/80 antibody (left) and red fluorescence from an anti-phosphorylated STAT3 antibody (middle) identified the F4/80-positive macrophages as expressing phosphorylated STAT3 when the images were superimposed (**arrowheads** at right). Scale bars: 50 μm (**A, B**); 10 μm (**insets**).

Localization of Phosphorylated STAT3 in Murine CNV

To examine the expression and tissue localization of phosphorylated STAT3 in murine CNV, CNV tissues were stained with an antibody against phosphorylated STAT3 together with isolectin B4 or an anti-F4/80 antibody, markers for vascular endothelial cells or macrophages, respectively. The immunohistochemical analyses for murine CNV showed phosphorylated STAT3 staining on isolectin B4-positive endothelial cells (Figure 3A) and F4/80-positive macrophages (Figure 3B).

Suppression of STAT3, but Not ERK1/2, Phosphorylation by IL-6R Signaling Blockade with MR16-1

STAT3 and ERK MAP kinase signaling cascades are two major pathways activated by IL-6/IL-6R via gp130. To define the signaling pathway involved in the treatment with MR16-1, we analyzed the ratios of protein levels of phosphorylated forms of STAT3 and ERK1/2 to total STAT3 and ERK1/2 in the RPE-choroid complex. STAT3 and ERK1/2 were significantly activated in the RPE-choroid complex by inducing CNV ($P < 0.05$; Figure 4, A–C). IL-6R signaling blockade by MR16-1 significantly suppressed STAT3, but not ERK1/2, phosphorylation in the RPE-choroid complex ($P < 0.05$; Figure 4, A–C). Importantly,

CNV was significantly suppressed by blocking JAK/STAT pathway with a JAK2 inhibitor AG490. AG490-treated mice at the dose of 0.1 or 1 $\mu\text{g/g}$ showed a significant decrease in the index of CNV volume ($404,976 \pm 114,524 \mu\text{m}^3$ for 0.1 $\mu\text{g/g}$, $309,966 \pm 57,126 \mu\text{m}^3$ for 1 $\mu\text{g/g}$) compared with vehicle-treated mice ($505,750 \pm 144,701 \mu\text{m}^3$) (Figure 4, D and E).

In Vivo Inhibition of Inflammatory and Angiogenic Molecules by IL-6R Signaling Blockade

To determine whether IL-6R signaling blockade affects inflammatory and angiogenic molecules related to the pathogenesis of CNV, mRNA expression of *ICAM-1*, *MCP-1*, and *VEGF* in the RPE-choroid complex was analyzed by semiquantitative RT-PCR (Figure 5A). mRNA expression of *ICAM-1*, *MCP-1*, and *VEGF* in the RPE-choroid complex was up-regulated by inducing CNV. IL-6R signaling blockade by systemic administration of MR16-1 substantially reduced mRNA expression of *ICAM-1*, *MCP-1*, and *VEGF* (both 164 and 120 isoforms). In addition, protein levels of ICAM-1, MCP-1, and VEGF in the RPE-choroid complex were higher in mice with CNV than in age-matched normal controls (Figure 5, B–D). IL-6R signaling blockade by MR16-1 significantly suppressed protein levels of ICAM-1 ($P < 0.01$), MCP-1 ($P < 0.05$), and VEGF ($P < 0.01$).

Figure 4. Suppression of STAT3, but not ERK1/2, activation by blocking of IL-6R signaling with MR16-1. **A–C:** Western blotting for phosphorylated and total levels of STAT3 and ERK1/2 in the RPE-choroid after photocoagulation. The graphs show the ratios of phosphorylated to total STAT3 (**B**) and ERK1/2 (**C**). STAT3 and ERK1/2 were significantly activated in the RPE-choroid complex by inducing CNV. IL-6R signaling blockade by MR16-1 (10 μg/g) significantly suppressed STAT3, but not ERK1/2, phosphorylation in the RPE-choroid complex. $n = 8$. $^{\dagger}P < 0.05$. **D and E:** Suppression of CNV by blocking STAT3 pathway with AG490. The graph shows the index of CNV volume (**D**). Flat-mounted choroids from vehicle-treated mice and AG490-treated mice (0.1 and 1 μg/g body weight) (**E**). Arrowheads indicate lectin-stained CNV tissues. $n = 20$ to 30. $^{**}P < 0.001$, $^{*}P < 0.01$. Scale bars = 50 μm.

In Vitro Inhibition of Phosphorylated STAT3 and Inflammatory Molecules by IL-6R Signaling Blockade

To confirm the MR16-1-induced suppression of STAT3 phosphorylation *in vivo* (Figure 4) and choroidal expression of various inflammatory and angiogenic molecules (Figure 5), we further performed *in vitro* analyses (Figure 6). IL-6 production was markedly induced by the treatment with LPS (data not shown). The ratios of phosphorylated to total STAT-3, significantly ($P < 0.01$) elevated by LPS application, were significantly ($P < 0.01$) suppressed by the application of MR16-1 in both b-End3 cells (Figure 6, A and B) and RAW264.7 macrophages (Figure 6, F and G).

We analyzed mRNA (Figure 6, C and H) and protein (Figure 6, D, E, and I) levels of ICAM-1 and MCP-1 in

b-End3 vascular endothelial cells (Figure 6, C–E) and VEGF in RAW264.7 macrophages (Figure 6, H and I). In b-End3 cells, mRNA (Figure 6C) and protein levels (Figure 6, D and E) of ICAM-1 and MCP-1, induced by the exposure to LPS, were significantly suppressed by the treatment with MR16-1 ($P < 0.05$). In RAW264.7 macrophages, mRNA (Figure 6H) and protein (Figure 6I) levels of VEGF, induced by LPS stimulation, were significantly suppressed by the treatment with MR16-1 ($P < 0.01$).

Effects of IL-6R Signaling Blockade on Macrophage Infiltration

As the cellular mechanism in the pathogenesis of CNV, infiltration of inflammatory cells including macrophages

Figure 5. *In vivo* effects of IL-6R blockade on choroidal expression of inflammatory and angiogenic molecules analyzed by RT-PCR (A) and ELISA (B–D). IL-6R blockade by the administration of MR16-1 significantly suppressed protein levels of ICAM-1 (B), MCP-1 (C), and VEGF (D). $n = 8$ to 10. * $P < 0.01$, † $P < 0.05$. N.S., not significant.

plays a critical role in its growth. We compared the area-adjusted number of macrophages, which was adjusted by the area of CNV, between mice treated with MR16-1 versus control IgG (Figure 7, A and B). MR16-1-treated mice at the dose of 10 or 100 µg/g showed a significant decrease in the number of F4/80-positive macrophages, compared with control IgG-treated animals ($P < 0.01$).

Discussion

The present study reveals, for the first time to our knowledge, several important findings concerning the role of IL-6 signaling in the development of CNV. First, CNV induction by laser treatment stimulated IL-6 expression in the RPE-choroid complex (Figure 1), and antibody-based blockade of IL-6R or genetic ablation of IL-6 led to significant suppression of CNV (Figure 2). Second, CNV generation was accompanied by STAT3 activation in choroidal endothelial cells and macrophages (Figure 3), and IL-6R blockade resulted in selective inhibition of STAT3, but not ERK1/2, phosphorylation (Figure 4). Consistently, pharmacological blockade of STAT3 pathway suppressed CNV (Figure 4). Third, the molecular and cellular mechanisms in the IL-6 signaling blockade included the inhibitory effects on inflammation-related molecules in the RPE-choroid complex (Figure 5) and in cultured endothelial cells and macrophages (Figure 6) and on macrophage infiltration into CNV (Figure 7).

Our current data demonstrate the critical role of IL-6 signaling in CNV, the pathogenesis of which has proven to be mediated by inflammation. This is supported by the previous reports showing the pathogenic role of IL-6 in inflammatory disease models including collagen-induced

Figure 6. *In vitro* effects of MR16-1 on phosphorylated STAT-3 (A, B, F, G), mRNA expression (C, H), and protein levels (D, E, I) of inflammatory and angiogenic molecules in b-End3 endothelial cells (A–E) and RAW264.7 macrophages (F–I). A and B: MR16-1 significantly suppressed STAT3 phosphorylation induced by LPS in b-End3 cells. $n = 6$. * $P < 0.01$. C: ICAM-1 and MCP-1 mRNA expression stimulated by LPS was substantially suppressed by MR16-1. D and E: MR16-1 significantly reduced protein levels of ICAM-1 and MCP-1. F and G: MR16-1 significantly suppressed STAT3 phosphorylation induced by LPS in RAW264.7 cells. $n = 6$. * $P < 0.01$. H: VEGF mRNA expression stimulated by LPS was substantially suppressed by MR16-1. I: Protein level of VEGF in RAW264.7 cells stimulated by LPS was significantly suppressed by MR16-1. $n = 8$. * $P < 0.01$, † $P < 0.05$.

murine arthritis²² and Th1 cell-mediated murine colitis.²³ IL-6/IL-6R binding-mediated gp130 dimerization is known to cause the activation of STAT3 and ERK MAP kinase pathways. Genetically altered mice with a point mutation in gp130, capable of activating STAT3, but not ERK MAP kinase, exhibited joint inflammation mimicking human rheumatoid arthritis,²⁴ suggesting significant contribution of STAT3 to subsequent inflammatory events. In the rat model of LPS-induced retinal inflammation, STAT3

Figure 7. Inhibitory effect of IL-6R blockade on macrophage infiltration into CNV. **A:** F4/80-positive macrophages (top) and PECAM-1-stained neovascularization area (bottom) were evaluated in murine CNV. **B:** The area-adjusted number of macrophages is shown in the graph. $n = 20$ to 23 . * $P < 0.01$.

was activated in astrocytes.²⁵ Recent immunohistochemical analyses for CNV tissues from patients with AMD revealed the expression of phosphorylated STAT3,²⁶ consistent with our data showing the activation of STAT3 in the murine model of laser-induced CNV (Figure 3). Indeed, CNV was significantly suppressed by blocking the JAK/STAT pathway with the JAK2 inhibitor (Figure 4). In accordance with these results, the IL-6R neutralization-induced reduction of CNV was mediated by inhibition of STAT3, but not ERK1/2, activation (Figure 4), suggesting a pivotal role of STAT3 as IL-6-induced intracellular signaling in CNV generation. The induction of CNV was shown to be associated with several signaling pathways mediated by angiotensin II type 1 receptor²⁷ and prorenin receptor (S Satofuka, A Ichihara, N Nagai, Y Oike, S Ishida, unpublished data), both of which are known to lead to the activation of ERK MAP kinase.^{28,29} These alternative pathways are likely to explain the present finding that IL-6R blockade did not suppress the phosphorylation of ERK1/2 (Figure 4).

As IL-6 signaling-mediated molecular and cellular mechanisms for promoting CNV, the present data showed that IL-6R blockade led to significant suppression of CNV-related molecules including ICAM-1, MCP-1, and VEGF *in vivo* (Figure 5) and *in vitro* (Figure 6) and of macrophage infiltration (Figure 7). Previous reports concerning the molecular mechanisms underlying CNV generation showed VEGF as a critical angiogenic factor.

VEGF was detected in both the experimental model of laser-induced CNV³⁰ and surgically excised CNV tissues from patients with AMD.³¹ Antibody-based blockade of VEGF led to significant suppression of experimental CNV.³ More recently, several *in vivo* experiments with genetically altered mice demonstrated significant roles of adhesion molecules and chemotactic factors including ICAM-1 and MCP-1,^{5,9} both of which are required for macrophage infiltration. Macrophages, the rich source of VEGF, facilitate the development of CNV.^{30,31} Collectively, the currently observed suppression of CNV by blocking IL-6R is probably attributable to the inhibition of multiple inflammatory steps including MCP-1-induced migration and ICAM-1-dependent adhesion of macrophages and subsequent macrophage-derived VEGF secretion. This is compatible with the recent data showing that IL-6R neutralization led to significant suppression of macrophage infiltration via STAT3 deactivation in the murine model of spinal cord injury³² and that STAT3 activation was required for IL-6-induced expression of ICAM-1³³ and VEGF.³⁴

A recently established neutralizing antibody against human IL-6R, tocilizumab, interacts with both membrane-bound and soluble forms of IL-6R, resulting in the blockade of IL-6-mediated signaling via IL-6R,³⁵ and is clinically applied to intractable inflammatory disorders including rheumatoid arthritis, Castleman's disease, and Crohn's disease.^{36–39} Rheumatoid arthritis, characterized by multiple joint inflammation with bone destruction, develops IL-6 production in synovial cells and macrophages. Application of the IL-6R neutralizing antibody led to significant decrease in the severity of the disease.^{36,37} In Castleman's disease, a rare lymphoproliferative disease associated with IL-6 overproduction in the swollen lymph nodes, the anti-IL-6R antibody administration was shown successfully to improve the clinical score of the disease.³⁸ Likewise, the anti-IL-6R antibody was reported to ameliorate the activity index of Crohn's disease, an inflammatory bowel disease characterized by intestinal stenosis and fistula.³⁹ These clinical trials have established the safety and efficacy of the IL-6R neutralizing antibody, showing the role of IL-6R-mediated signaling in various human inflammatory diseases.

Currently, anti-VEGF therapy including pegaptanib and ranibizumab is applied for the treatment of AMD complicated by CNV.⁴⁰ Because the therapeutic intervention for blocking VEGF tends to be limited to the advanced stage when the visual function is irreversibly affected by CNV formation, an alternative early treatment is thought to be required targeting inflammation as an antecedent event leading to neovascularization. In the present study, IL-6R blockade resulted in significant suppression of the *in vivo* (Figure 5) and *in vitro* (Figure 6) expression of VEGF, suggesting IL-6 as an upstream inflammatory stimulant for VEGF. Epidemiological risk factors for AMD include several components of the metabolic syndrome,^{41–45} the pathogenesis of which has proven to be mediated by silent, chronic inflammation.^{46,47} Importantly, a recent prospective cohort study concerning the relationship of AMD with several cardiovascular biomarkers has revealed that increased serum

levels of IL-6 and C-reactive protein are independently associated with progression of AMD.¹⁷ Indeed, our new data show that serum levels of C-reactive protein are increased in the murine model of laser-induced CNV (T Koto, N Nagai, H Mochimaru, T Kurihara, K Izumi-Nagai, S Satofuka, H Shinoda, K Noda, Y Ozawa, M Inoue, K Tsubota, Y Oike, S Ishida, manuscript submitted, 2006). C-reactive protein, induced by IL-6 in the liver, is known to activate the classical complement pathway in the injured tissues. Interestingly, complement fragments C3a and C5a accumulated beneath the RPE in human AMD and murine CNV have been shown to induce VEGF expression, promoting CNV generation.⁴⁸ Reasonably, IL-6R blockade, which may inhibit not only inflammatory neovascularization in the eye but also improve the systemic background predisposing to AMD, is likely to be a novel therapeutic strategy as a preventive, early, and additive treatment for AMD. A large-scale, prospective and randomized clinical trial is needed to validate the inhibitory effect of IL-6R blockade on CNV.

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