Osteopontin Is Proinflammatory in Experimental Autoimmune Uveitis

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PURPOSE. Osteopontin (OPN) has been implicated in inflammatory and wound-healing processes. Increased OPN mRNA levels have been reported in experimental autoimmune uveitis (EAU), but the function of OPN in the inflamed eye is unknown. The purpose of this study was to investigate the role of OPN in the pathogenesis of EAU.

METHODS. EAU was induced in OPN-null and wild-type (WT) mice by immunization with interphotoreceptor retinoid-binding protein (IRBP). Immunofluorescence experiments were performed to identify OPN-positive cells in WT mice. Disease incidence, serum IRBP antibody levels, vitreous infiltrates, retinal granulomas, and lymphocyte proliferation were assessed in OPN-null and WT mice. To determine whether OPN could induce an EAU-like condition, purified OPN and OPN fragments were injected intraocularly into WT mice and vitreous infiltrates were characterized and quantified.

RESULTS. In WT EAU-positive eyes, cell types with increased OPN immunoreactivity were identified as F4/80-positive macrophages/microglia and CD4-positive T cells. OPN-null mice manifested attenuated disease with decreased vitreous infiltrates, fewer granulomas, less lymphocyte proliferation, and lower serum IRBP antibody levels. Exogenous full-length OPN, as well as N- and C-terminal fragments, induced leukocyte infiltration and retinal folding, with some similarities to EAU.

CONCLUSIONS. The results demonstrate that OPN is proinflammatory in EAU and may be important for recruitment and activation of leukocytes in retinal inflammation. (Invest Ophthalmol Vis Sci. 2006;47:4435–4443) DOI:10.1167/iovs.06-0064

Ocular inflammatory diseases with presumed autoimmune origins, generally known as uveitis, often lead to irreversible destruction of retinal tissues and are responsible for 15% of visual impairment in the United States.¹² Systemic diseases that affect the eye include Behçet’s disease and Vogt-Koyanagi-Harada syndrome, whereas sympathetic ophthalmia and birdshot retinochoroidopathy are diseases specific to the eye.³ Experimental autoimmune uveitis (EAU) is a well-characterized animal model of human ocular inflammation that is induced by injection of an autoantigen, such as interphotoreceptor retinoid-binding protein (IRBP), or adoptive transfer of antigen-primed T cells.²–⁴ In genetically susceptible mice, changes associated with EAU peak 14 days after immunization.⁵–⁷ Histologic hallmarks of EAU include leukocyte infiltration, retinal granulomas, retinal folding/detachment, and eventually, thinning of the retina.⁵–⁷ Disease progression in EAU is largely mediated by CD4-positive T cells,¹⁰–¹² but is exacerbated by infiltrating activated macrophages.¹³ In addition, recent work indicates that activated retinal microglia may initiate the inflammatory response by recruiting leukocytes.¹⁴,¹⁵

In a cDNA array analysis of EAU eyes, Foxman et al.¹⁶ identified several genes that were elevated in disease, including osteopontin (OPN). However, the cells expressing OPN, and its function in the inflamed eye are unknown.

First described as a protein secreted by transformed mammalian cells,¹⁷ OPN has since been reported in a variety of normal and pathologic tissues, and evidence to support its importance in the immune response is growing.¹⁸ Also known as early T-lymphocyte activation (Eta)-1, OPN is a pleiotropic, phosphorylated glycoprotein that can be matrix associated or act as a cytokine.¹⁹,²⁰ OPN is involved in inflammation²¹,²² and is known to enhance the Th1 response and decrease the Th2 response.¹⁹ It is produced by macrophages and T cells and is also chemotactic and induces activation of these same cells.²³–²⁵ OPN downregulates inducible nitric oxide synthase in macrophages, conferring antioxidant effects.²⁶ Furthermore, OPN can bind to and sequester complement factor H and protect cells from complement-mediated cell lysis.²⁷ OPN-null mice exhibit immune response defects in Th1 response, granuloma formation, and wound healing.¹⁹,²⁸,²⁹ Increased expression of OPN in vivo has been correlated with sepsis, metastatic cancer, heart failure, and tissue injury,²⁵,²⁶ particularly in the central nervous system (CNS).

Although OPN is expressed in the normal developing and mature nervous system, including in retinal neurons,²⁷–²⁹ its expression has also been observed in macrophages and/or microglia after spinal cord injury,³⁰ kainate-induced excitotoxicity in the brain,³¹ and global forebrain ischemia.⁴⁰ In experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis, OPN-null mice show attenuated disease with decreased infiltrating immune cells.⁴¹–⁴⁴ These data point to OPN as a potentially significant molecule in a variety of neuroinflammatory diseases.

In this study, we investigated the involvement of OPN in EAU. First, OPN protein expression was characterized before and after the development of EAU. Second, disease progression was analyzed in OPN-null and WT mice. Finally, we determined whether exogenous OPN was sufficient to replicate any immune-related diseases observed in EAU. Our results show that...
OPN immunoreactivity was increased after induction of EAU and that infiltrating macrophages and T cells, as well as resident activated microglia and retinal ganglion cells (RGCs), express OPN protein. OPN-null mice with EAU showed decreased disease incidence and attenuated disease characterized by decreased serum antibody against IRBP, diminished lymphocyte proliferative response, and significantly decreased vitreous infiltrates (T cells and macrophages) and retinal granulomas. Exogenous OPN was sufficient to cause retinal inflammation-like disease in normal eyes, characterized by leukocyte infiltration and retinal folding and detachment. Our results indicate that OPN is proinflammatory in retinal inflammation via activation and recruitment of immune cells.

**MATERIALS AND METHODS**

**Animals**

Experiments were performed on C57BL/6 x 129/SvJ mice with a targeted disruption of the osteopontin (spp1) gene, generated as previously described by Liaw et al.29 OPN-null mice were rederived and then backcrossed with C57BL/6 mice for eight generations for genetic homogeneity (mice were a generous gift from Ronald Goldfarb; Sophe- rion Therapeutics, Princeton, NJ). Genotyping was performed via polymerase chain reaction (PCR) amplification of WT and mutant alleles from DNA purified from tail samples (DNeasy Kit; Qiagen, Valencia, CA). Primers and PCR parameters were as described previously.29 Female mice, approximately 4 months of age, were housed in patho- gen-free conditions. Sterile water and autoclaved rodent chow were provided ad libitum. All studies were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Santa Barbara.

**EAU Induction**

EAU was induced in WT and OPN-null mice by subcutaneous immunization (0.1 mL at the tail base and 0.05 mL in each thigh) with 150 µg IRBP (Veritas, Inc., Rockville, MD) emulsified in complete Freund’s adjuvant with 2.5 mg/mL *Mycobacterium tuberculosis* H37RA. Pertussis toxin (0.5 µg; Sigma-Aldrich, St. Louis, MO) was given concurrently via intraperitoneal injection. Mice were euthanatized at 14 or 21 days after immunization. One eye was processed for histology and disease scoring, one eye was processed for immunostaining, draining lymph nodes were collected for lymphocyte proliferation and cytokine assays, and blood was collected intracardially for IRBP antibody quantification.

**Histologic Assessment of Disease**

Enucleated eyes were fixed in 4% glutaraldehyde in 0.15 M phosphate buffer for 1 hour at room temperature, followed by fixation in 10% formalin overnight at room temperature. Samples were embedded in methacrylate resin, sectioned through the pupillary–optic nerve plane, and stained with hematoxylin and eosin (H&E) for histologic evaluation by a masked investigator. Scoring was performed as described by Caspi et al. Briefly, severity of disease was scored on a scale of 0 (no disease) to 4 (maximum disease). EAU was defined as positive (EAU score ≥ 0.5) when inflammatory cell infiltration was observed in the choroid, ciliary body, and retina. Perivascular or vitreous infiltration of leukocytes was scored as 1. Presence of granuloma formation, vascularitis, retinal folds, serous detachment, photoreceptor loss/damage was graded as 2. Subretinal neovascularization and presence of Dallen–Fuchs-like nodules (subretinal granulomas) were scored as 3, with a higher score of 4 assigned if the number and size of the lesions were more substantial.

**Lymphocyte Proliferation Assay**

This assay was performed as described previously.45 Briefly, draining lymph nodes were harvested 14 days after IRBP immunization and pooled within each group (five mice per genotype). A single-cell suspension of lymphocytes was collected and stimulated with graded doses of IRBP or with phytohemagglutinin (PHA; Murex Diagnostics, Darford, UK). After an incubation of 72 hours at 37°C, cells were pulsed with [3H]thymidine. Sixteen hours later, the incorporated radioactivity was measured by a scintillation counter. Data are expressed as mean counts per minute (cpm).

**IRBP Antibody Assay**

Serum levels of antibody against IRBP from mice 14 days after immunization were quantified using enzyme-linked immunosorbent assay (ELISA), as described previously.46 Briefly, wells were coated with 0.3 µg IRBP for 1 hour at 37°C. Serially diluted serum samples of each mouse were added to the wells. Normal mouse serum (NMS) was used as a control. After incubation at 37°C for 1 hour to allow antibody to bind antigen, the sera were removed and the wells were washed. Bound antibody was detected with peroxidase-conjugated goat anti-mouse IgG (ICN Biomedicals, Aurora, OH) and application of O-phenylenediamine substrate in 0.1 M citrate buffer. Absorbance was read at 405 nm and mean optical density (OD ± SEM) was calculated for each group, at each dilution.

**FIGURE 1.** OPN immunoreactivity in EAU eyes. Confocal micrographs of normal (A, B) and EAU (C–F) eyes 21 days after immunization are shown. Cryosections were probed with antibodies against OPN (red) and GFAP (green). Red arrowheads: OPN immunoreactivity in RGCs; white arrows: additional OPN immunoreactivity in EAU. Note increased GFAP immunoreactivity in EAU eyes (D, F). Vit, vitreous; RPE, retinal pigment epithelium. Scale bar, 50 µm.
Intravitreal Injection of Osteopontin

Mice were anesthetized by intraperitoneal (IP) injection of Avertin (1.75% tribromoethanol and 1.75% tertiary amyl alcohol) at a dose of 0.017 mL/g body weight. A topical anesthetic, 0.5% proparacaine hydrochloride (Falcon Pharmaceuticals, Fort Worth, TX), was administered to each eye before intravitreal injection. Eyes were first punc-

tured by microscope (BX-60; Olympus). 4

duced N- and C-terminal OPN fragments47 (gift of Lucy Liaw, Maine Medical Center Research Institute, Scarborough, ME) were also intra-

vitrally injected. This amount was used in previous studies,48 and was chosen to effect a gross excess (~100 fold) over endogenous levels of OPN present in the vitreous (based on OPN ELISA results). For each mouse, one eye received an OPN or mouse serum albumin (MSA) injection, and the contralateral eye received a saline injection. All solutions had a final pH of 7.4. Mice were euthanatized at 1 day and 5 days after injection. Enucleated eyes were processed as will be de-

scribed for immunostaining and histologic examination.

Immunofluorescence

Enucleated eyes were immersion-fixed in 4% paraformaldehyde at 4°C and cryoprotected and sectioned with deionized water, and coverslipped, and digital images were cap-

tured by microscope (MRC 1024; BioRad, Hercules, CA, and streptavidin-Cy5 (1:200; Jackson Immunoresearch, West Grove, PA), and streptavidin-Cy5 (1:200; Jackson Immunoresearch, Golden, CO) was used to identify Müller glial cells. Specificity of the OPN antibody was confirmed by absence of signal in OPN-null mice sections. Fluorescently conjugated secondary antibodies were applied to sections for 30 minutes: AlexaFluor donkey anti-goat IgG 546, AlexaFluor donkey anti-rat IgG 488 (1:100; Invitrogen-Molecular Probes, Eugene, OR), donkey anti-rabbit IgG-Cy5 (1:400; Jackson Immunoresearch, West Grove, PA), and streptavidin-Cy5 (1:200; Jackson Immunoresearch). 4',6'-Diamino-2-phenylindole (DAPI; 5 μg/mL) was added to some sections to stain cell nuclei for vitreous infiltrate quantification. To control for background due to nonspecific binding of secondary antibodies, primary antibodies were omitted from adja-

cent tissue sections. In all experiments, negative control cells showed no immunofluorescence. Cryosections were coverslipped with anti-

fade mounting medium (Prolong; Invitrogen-Molecular Probes). Im-

ages were captured by microscope (MRC 1024; BioRad, Hercules, CA, or Fluoview 500; Olympus, Tokyo, Japan) laser scanning confocal microscope. Some sections were stained with methylene blue, washed with deionized water, and coverslipped, and digital images were cap-

tured by microscope (BX-60; Olympus).

Quantification of Vitreous Infiltrates

DAPI-positive cells in the vitreous were counted for total vitreous infiltrates. Infiltrating macrophages were identified as F4/80-positive cells and infiltrating T cells were identified as CD4-positive cells. For
each eye, photographs of two fields per section (peripheral and central retina) were taken (at least three sections/eye were analyzed) using monochrome ASA 400 film (T-Max; Eastman Kodak, Rochester, NY) on a epifluorescence microscope (PM-3; Carl Zeiss Meditec., Inc., Thornwood, NY) equipped with a 35-mm camera (FX-35A; Nikon, Tokyo, Japan). Cells were counted visually and data are represented as the mean number of cells per frame. Some sections were imaged on the confocal microscope, as described earlier.

Quantification of Retinal Granulomas
H&E-stained sections (at least four sections/eye) were analyzed on a light microscope (Carl Zeiss Meditec., Inc.), and retinal granulomas were visually counted using a double-blind procedure. Data are represented as the mean number of granulomas per section. Images of representative sections were captured digitally (BX-60 microscope; Olympus).

Statistical Analysis
Differences between mean values for OPN-null and WT mice in mean EAU score, lymphocyte proliferation assay, vitreous infiltrate quantification, and retinal granuloma quantification were analyzed using Student’s two-tailed t-test. Analysis of disease incidence was performed by χ² test, and data are represented as the percentage incidence of EAU (percentage of animals positive). P < 0.05 was considered significant.

RESULTS
OPN Immunoreactivity in EAU
To characterize OPN protein expression in the eye during EAU, retinal sections from WT mice immunized with IRBP (and from nonimmunized control mice) were probed with antibodies against OPN and GFAP. In the control retina, OPN immunoreactivity was found in a subset of retinal ganglion cells (RGCs; Fig. 1A, red arrowheads). GFAP immunoreactivity was found in astrocytes and Müller glial end feet near the inner limiting membrane (Fig. 1B). In EAU eyes, OPN immunoreactivity persisted in RGCs at intensities similar to cells of control eyes (Figs. 1C, 1E, red arrowheads). Additional OPN-positive cells were observed throughout the neural retina, as well as in the vitreous and choroid (Fig. 1E, white arrows). Moreover, GFAP immunoreactivity in Müller glial cells increased dramatically in EAU eyes (Figs. 1D, 1F).

To determine whether OPN-positive cells in EAU eyes were immune cells, we probed sections with antibodies against OPN, IB4, and CD4. OPN immunoreactivity colocalized with a subset of IB4-positive vitreous-infiltrating macrophages (Figs. 2A–C, arrows) and putative resident microglia (Figs. 2A–F, arrowheads), which were identified by their characteristic amoeboid morphology and their location throughout the neural retina. Retinal granulomas, in particular, showed a high number of OPN-positive and IB4-positive cells. Note the surrounding OPN-positive activated microglia (Figs. 2D–F, arrowheads). OPN immunoreactivity also colocalized with a subset of CD4-positive T cells in the vitreous (Figs. 2G–I, arrows).

EAU in OPN-Null and WT Mice
To address the function of OPN in EAU, disease was induced in OPN-null mice and compared to WT mice. EAU incidence

![Graph A](image)

**Figure 3.** EAU in OPN-null and WT mice. (A) EAU incidence. Mice were assessed 14 days after immunization. For quantification of EAU incidence, mice with a score ≥ 0.5 (scale, 0–4) were considered EAU-positive. P < 0.05, n = 10 WT mice, 8 OPN-KO mice. (B) Analysis of serum levels of IRBP antibody. Sera from individual OPN-null (KO) and WT mice were collected 14 days after immunization and analyzed via direct ELISA to determine anti-IRBP antibody levels. NMS was used as a control. Data are presented as mean OD ± SEM of each group, at each time point (n = 10 WT mice, 8 OPN-null mice). WT and null mice were significantly different at each dilution (P < 0.01). (C) Lymphocyte response to IRBP antigen. Lymphocytes from draining lymph

nodes of the immunization site were isolated 14 days after immunization, and the proliferative response to graded doses of IRBP antigen was assessed. Data are expressed as mean cpm of triplicate cultures of pooled lymph node cells from five mice per genotype. Student’s two-tailed t-test was used for statistical analysis. Error bars represent the SEM (**P < 0.001, *P < 0.005, #P < 0.02).
was decreased by 37.5% in OPN-null mice (Fig. 3A; \( P < 0.05 \)). Mean EAU scores in OPN-null mice were also lower than WT control mice, but the difference was not statistically significant (OPN null \( P = 0.05 \), WT \( P = 0.42 \)). A distribution plot of disease scores also showed an attenuated disease trend in the OPN-null mice but the results were not statistically significant via a nonparametric Mann-Whitney test. However, additional quantitative analyses of disease parameters showed significant differences between OPN-null and WT mice.

The immune response of OPN-null mice during EAU was quantified and compared to WT mice. OPN-null mice showed a moderate but significant decrease of serum levels of antibody against IRBP compared with WT mice (Fig. 3B). To assess further the immune response, primed T cells isolated from draining lymph nodes were stimulated with IRBP antigen in vitro, and their proliferative response was quantified (Fig. 3C). T cells from OPN-null mice had a decreased proliferative response not only to IRBP antigen but also to phytohemagglutinin (PHA), the positive control. Decreases in mean cpm for OPN-null lymphocytes were: PHA 1 g/mL, 30%; IRBP 0.3 g/mL, 40%; IRBP 3 g/mL, 46%; and IRBP 30 g/mL, 22%.

Differences between OPN-null and WT mice were also observed when eye sections were analyzed for infiltrating cells. In disease-score matched retinal sections, OPN-null mice had consistently fewer cells infiltrating the vitreous than did WT mice (Figs. 4A, 4B). Quantification of OPN-null cells positive for DAPI, CD4, and F4/80 showed significant decreases in all three categories of vitreous infiltrates: DAPI-positive cells, 47%; CD4-positive cells, 65%; and F4/80-positive cells, 40% (Fig. 4C). This decrease in inflammatory cell infiltration is also apparent in Figures 1 and 2. In addition, the mean number of retinal granulomas per section was decreased by 52% in the OPN-null mice (Fig. 4D).

**Effect of Exogenous OPN**

To determine whether OPN was sufficient to cause an inflammatory response in normal mouse eyes, purified mouse OPN was injected intravitreally, and eyes were compared to controls injected with saline or mouse serum albumin. In OPN-injected
eyes, the layered retinal organization was severely disrupted 5 days after injection, with retinal detachment, folding, and vitreous infiltrates similar, but not identical, to that observed in EAU (Figs. 5C, 5D; compare to Figs. 1, 2). Saline-injected eyes appeared normal (Figs. 5A, 5B). To identify vitreal cells, immunofluorescence was performed using antibodies against OPN, F4/80, CD4, and GFAP. The 5 days after OPN injection, eyes exhibited a dramatic influx of OPN- and F4/80-positive macrophages, as well as F4/80-positive activated microglia, a subset of which was positive for OPN (Figs. 6E, 6F). Also, GFAP expression was increased in the OPN-injected eye (Fig. 6G), similar to the expression observed in EAU. In contrast, saline-injected eyes appeared normal (Figs. 6A–D). CD4 immunoreactivity was not observed in either OPN or saline-injected eyes.

Based on previous work reporting chemotactic and activating functions of proteolytically cleaved OPN fragments, we next tested whether OPN fragments have immunologic activity in the normal mouse eye. OPN N- and C-terminal fragments corresponding to those produced in vivo by MMP cleavage50 were injected intravitreally and examined 1 day later. C-terminal OPN induced a significant degree of F4/80-positive macrophage infiltration, similar to full-length OPN (Figs. 7B, 7C). N-terminal OPN appeared to show a reduced degree of F4/80-positive macrophages (Fig. 7D). Some microglia appeared to be activated with full-length and C-terminal OPN (Fig. 7C), but none were observed with N-terminal OPN. To exclude the possibility of a nonspecific response to intravitreal protein injection, 250 ng of mouse serum albumin was injected into the vitreous of control animals. Only a small number of F4/80-positive macrophages were observed in the mouse serum albumin control eyes, which may be due to the physical trauma of injection, as similar results were observed in saline-injected eyes.

**DISCUSSION**

This study examined the expression and function of OPN protein in EAU and demonstrated that (1) OPN protein was increased in EAU; (2) macrophages/microglia and T cells accounted for the increased OPN expression, whereas expression from RGCs remained unchanged; (3) OPN-null mice had decreased EAU incidence and an attenuated immune response; and (4) exogenous OPN caused abnormalities similar to EAU. These data show that OPN is expressed by immune cells in the eye and suggest that OPN functions to activate and recruit leukocytes during EAU.
This finding is consistent with previous work showing OPN expression by retinal microglia. Although OPN expression in brain microglia has been described, to our knowledge, this is the first report of OPN expression by retinal microglia. Recent evidence indicates that microglial activation precedes leukocyte infiltration in EAU and may play a key role in initiating the inflammatory cascade. Like macrophages, activated microglia release proinflammatory cytokines and are involved in antigen presentation to T cells. Microglia-expressed OPN may be a chemoattractant/activating factor for the initial infiltrating leukocytes in the EAU eye. OPN expression was observed on a subset of activated microglia/macrophages and T cells, similar to the case in cardiac injury, suggesting that there may be regulatory differences and/or a graded response in OPN-mediated leukocyte activation. Further work is needed to determine the specific function of microglia-expressed OPN.

To examine whether absence of OPN could minimize EAU disease, OPN-null mice were induced with EAU and evaluated. OPN-null mice exhibited decreased disease incidence and attenuated disease characterized by decreases in lymphocyte response, vitreous infiltration, and retinal granuloma frequency compared to WT. It is of note that the capacity to develop EAU is affected by OPN deficiency to a lesser extent than that to develop EAE. This observation suggests that there are significant differences between the pathogenic mechanisms of these two experimental autoimmune diseases.

OPN activates B cells, T cells, and macrophages and it is in this activated state that leukocytes function optimally. In OPN-null mice with EAU, serum IRBP antibody levels were decreased and IRBP-primed lymphocytes showed a diminished proliferative response to IRBP antigen. Even PHA, a potent T-cell mitogen, resulted in a decreased proliferative response from OPN-null lymphocytes, suggesting that T cells cannot be optimally activated in the absence of OPN. The decrease in CD4-positive T cells in the vitreous of OPN-null eyes with EAU may reflect defects in T-cell chemotaxis into the eye, in addition to activation. Further, decreases in F4/80-positive macrophages in vitreous infiltrates and retinal granulomas in OPN-null mice with EAU may indicate a deficiency in macrophage chemotaxis and/or activation.

One possible explanation for the decreased vitreal leukocytes and retinal granulomas observed in OPN-null eyes after EAU induction is that OPN-null mice may have a phenotypically decreased number of circulating leukocytes. To examine this possibility, hematologic analysis of normal OPN-null and WT mice was performed. We observed no difference in the absolute number of circulating lymphocytes and monocytes between OPN-null and WT mice (data not shown). We note that the absence of OPN did not completely preclude leukocyte infiltration, which is not surprising, since multiple cytokines are involved in the inflammatory response. Moreover, compensatory immune response mechanisms in OPN-null mice may occur, similar to that observed in IL-6 null mice. However, the magnitude of the effects on infiltrating leukocytes (nearly 50% reduction in the OPN-null mice) suggests that OPN may be an essential cytokine in leukocyte recruitment during EAU.

If the absence of OPN diminishes inflammation, can exogenous OPN replicate an EAU-like condition in the normal eye? During severe retinal inflammation, the blood–retina barrier is breached and infiltrating leukocytes are found in the vitreous, the vitreal surface of the inner limiting membrane, the choroid, or near blood vessels. Increased OPN immunoreactivity in RGCs did not appear to change compared with control eyes. This indicated that the increased OPN mRNA detected in EAU eyes was due to cellular sources other than RGCs.

During severe retinal inflammation, the blood–retina barrier is breached and infiltrating leukocytes are found in the vitreous, the vitreal surface of the inner limiting membrane, the choroid, or near blood vessels. Increased OPN immunoreactivity in EAU was observed in these same areas and double-labeling studies identified OPN-positive cells as IB4-positive macrophages and CD4-positive T cells in the retina and vitreous. This finding is consistent with previous work showing that OPN is expressed by macrophages and T cells. OPN also promotes chemotaxis of macrophages and T cells and acts as a cytokine to activate leukocytes. Therefore, it is possible that the OPN expressed by infiltrating leukocytes in EAU promotes chemotaxis of additional leukocytes into the affected eye and activates leukocytes until the inciting antigen has been cleared from the retina.

OPN may also be involved in leukocyte migration in EAU eyes. In some IB4-positive macrophages and CD4-positive T cells, the OPN expression pattern appeared intracellular. This likely reflects Golgi localization of OPN destined to be secreted as an extracellular matrix protein, which may provide an adhesive substrate for immune cell migration. Alternatively, an intracellular form of OPN has been described and is thought to be a component of a CD44-ERM (ezrin/radixin/moesin) complex involved in migration of activated macrophages.

OPN immunoreactivity was also observed in putative IB4-positive retinal microglia. Although OPN expression in brain microglia has been described, to our knowledge, this is the first report of OPN expression by retinal microglia. Recent evidence indicates that microglial activation precedes leukocyte infiltration in EAU and may play a key role in initiating the inflammatory cascade. Like macrophages, activated microglia release proinflammatory cytokines and are involved in antigen presentation to T cells.

FIGURE 7. Vitreous infiltrates after injection of OPN fragments. Confoocal micrographs of eyes 1 day after injection of OPN are shown. Cryosections were probed with antibodies against F4/80. Control eye injected with an equivalent amount of mouse serum albumin show few F4/80-positive cells (A), which is similar to results observed in saline-injected eyes. Full-length OPN (B) and OPN C-terminal (C) induced a significant amount of F4/80-positive infiltrates in the vitreous, whereas N-terminal OPN (D) induced a milder infiltration. (B) Arrowheads: activated microglia; open arrowhead: choroidal macrophage. Vit, vitreous; RPE, retinal pigment epithelium. Scale bar, 50 μm.

Increased OPN mRNA has been detected in EAU eyes, which led to our investigation of the specific cell types in the EAU eye that express OPN. In normal rodent eyes, OPN is expressed in the RGCs. The function of OPN in RGCs is unknown and requires further investigation. There were no obvious differences in retinal morphology between normal OPN-null and WT eyes (Figs. 4D, 4E). In EAU eyes, the level of OPN immunoreactivity in RGCs did not appear to change compared with control eyes. This indicated that the increased OPN mRNA detected in EAU eyes was due to cellular sources other than RGCs.

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positive infiltrates were not observed in the OPN-injected eyes. This suggests that, in the absence of an immunogenic antigen and subsequent T-cell activation, local activity of OPN can mediate macrophage recruitment and microglia activation in an acute immune response. Thus, exogenous OPN can recapitulate some, but not all, characteristics of EAU progression.

OPN may be cleaved by thrombin or matrix metalloproteinase (MMP)-3 or -7, to generate two OPN fragments of roughly the same size that have been shown to retain biological functions. This may be relevant to EAU, as both macrophages and T cells express MMPs to enable their chemotaxis through tissues, and MMP-7 was shown to increase in EAU retinas. We found that C-terminal OPN induced F4/80-positive macrophage infiltration, similar to that observed with full-length OPN, whereas N-terminal OPN appeared to induce macrophage infiltration to a slightly lesser degree. Some microglia appeared to be activated with full-length and C-terminal OPN, but none were observed with N-terminal OPN. Thus, it is possible that the observed proinflammatory effect of OPN in EAU may be additive, with increased expression by immune cells, followed by proteolytic processing of the active full-length protein into active fragments.

In summary, these data implicate OPN as an important proinflammatory regulator of the immune mechanisms underlying retinal inflammation.

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**References**


