# Oncomodulin links inflammation to optic nerve regeneration

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**The inflammatory response that accompanies central nervous system (CNS) injury can affect neurological outcome in both positive and negative ways. In the optic nerve, a CNS pathway that normally fails to regenerate when damaged, intraocular inflammation causes retinal ganglion cells (RGCs) to switch into an active growth state and extend lengthy axons down the nerve. The molecular basis of this phenomenon is uncertain. A prior study showed that oncomodulin (Ocm), a $Ca^{2+}$-binding protein secreted by a macrophage cell line, is a potent axon-promoting factor for RGCs. However, it is not known whether Ocm contributes to the physiological effects of intraocular inflammation in vivo, and there are conflicting reports in the literature regarding its expression and significance. We show here that intraocular inflammation causes infiltrative cells of the innate immune system to secrete high levels of Ocm, and that agents that prevent Ocm from binding to its receptor suppress axon regeneration. These results were verified in different strains, species, and experimental models, and establish Ocm as a potent growth-promoting signal between the innate immune system and neurons in vivo.**

retina | trophic factor

The response of the innate immune system to neural injury can affect functional outcome in multiple ways (1–3). In the peripheral nervous system, macrophages clear debris and help transform neurons into an active growth state (1, 4). The innate immune response in the central nervous system (CNS) is mediated by resident microglia, astrocytes, and infiltrating macrophages and neutrophils that can release numerous anti- and pro-inflammatory cytokines, chemokines, growth factors, and other signals. Although some of these factors are toxic to neurons, inflammation has been shown to promote axon regeneration and/or enhance cell survival in the optic nerve (5–9), dorsal roots (10, 11), and spinal cord (12, 13, 14) after injury. In the optic nerve, a CNS pathway that normally shows little capacity to regenerate when injured, peripheral nerve implants into the eye, lens injury, or intraocular injections of zymosan all cause macrophages to enter the eye and stimulate retinal ganglion cells (RGCs) to regenerate lengthy axons beyond the injury site (7, 8, 15). In addition, if RGCs exposed to the effects of inflammation are permitted to grow through a peripheral nerve graft or are transfected with genes to counteract cell-extrinsic inhibitors of growth, thousands of axons extend over long distances (8, 9, 16).

The mechanisms that link intraocular inflammation to optic nerve regeneration are unknown. We previously showed that an immortalized macrophage cell line secretes a small $Ca^{2+}$-binding protein, oncomodulin (Ocm), which enhances the ability of RGCs to regenerate axons in culture and in vivo (8, 17). It is not known, however, whether Ocm is expressed by macrophages physiologically or contributes to inflammation-induced regeneration in vivo. Injection of other trophic factors has also been shown to stimulate regeneration in the optic nerve (18–20), and one group reported that they were unable to detect Ocm in the eye after inducing inflammation and that a partial depletion of macrophages did not attenuate regeneration following lens injury (21). To understand the mechanistic links between inflammation and CNS regeneration, we have used several approaches to investigate the expression and functional role of Ocm. Our results establish Ocm as a potent signaling molecule between the immune system and neurons and as the principal mediator of inflammation-induced regeneration in vivo.

## Results

**Ocm Levels Increase Sharply after Inducing Inflammation.** To examine infiltrative cells that enter the eye after injuring the lens, we collected the aqueous/vitreous fluid (A/V) from the posterior chamber of the eye and either spread a standard volume onto slides (for cell counts and immunostaining) or sedimented the cells for mRNA and protein analyses. The number of cells expressing the macrophage marker CD68 increased markedly within a day of inducing inflammation and continued to rise over the next few days (Fig. 1*A*). Quantitative real-time PCR (TaqMan or SYBR Green PCR) showed that infiltrative cells expressed appreciable levels of Ocm mRNA on day 1 and somewhat less on days 3 and 7 (Fig. 1*B*). Unlike the infiltrative cells of the A/V, the retina expressed only low levels of Ocm mRNA and showed a relatively small increase relative to baseline following nerve injury and inflammation (Fig. 1*C*). Based upon Ocm mRNA standards, we found that the entire retina expressed only 7% as much Ocm mRNA as the 5,000 or so cells sedimented from the vitreous on day 1. Immunohistochemistry carried out with an affinity-purified antibody to the N terminus of Ocm showed Ocm to be concentrated in intracellular vesicles of ED1-positive cells (Fig. 1*D*; see Fig. 2*C* for the specificity of the antibody). Quantitatively, 96% of live cells present in the A/V were ED1-positive, and of these, 78% were Ocm-positive.

The association of Ocm with infiltrative cells was further demonstrated by Western blots (Fig. 1*E*). Inducing intravitreal inflammation by either lens injury (LI) or by injecting zymosan (Zy) into the eye resulted in similar elevations of Ocm. Despite expressing only low levels of the mRNA, the retina also showed a large increase in Ocm protein after LI or Zy (Fig. 1*F*). Based on the intensity of the Ocm band relative to that of recombinant Ocm (4 ng), the fraction of the samples run on the gels

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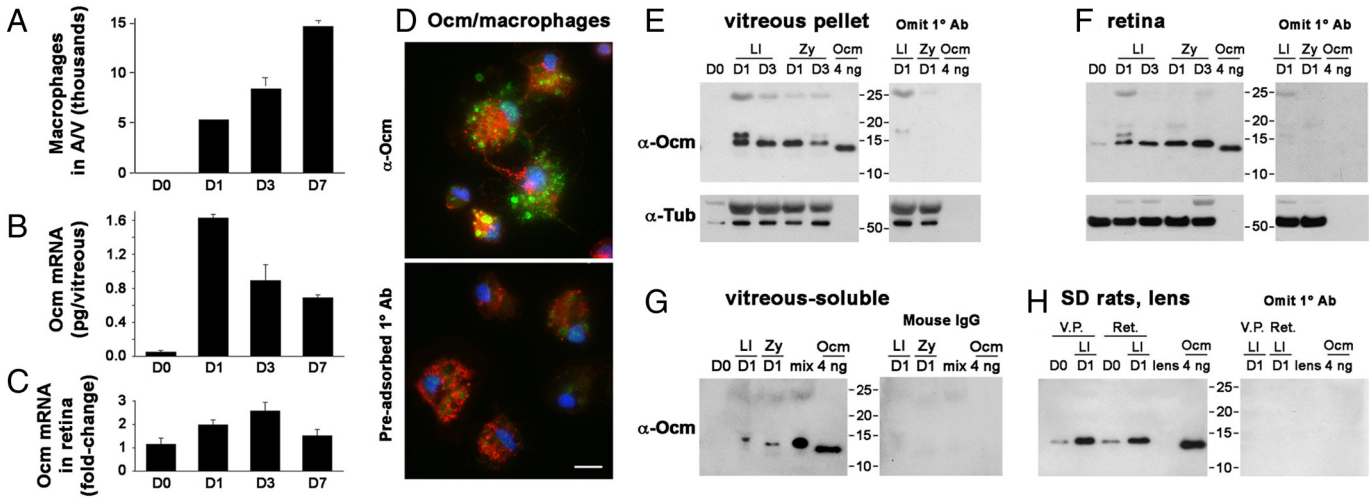


**Fig. 1.** Oncomodulin (Ocm) levels increase after lens injury. (*A*) Macrophage accumulation in the aqueous/vitreous humor (A/V) after lens injury. (*B*) Ocm mRNA in cells sedimented from the A/V. Each data point represents 3–4 biological replicates ($n$ = 6–16 cases/sample). Results are quantified based on Ocm mRNA standards. (*C*) Ocm mRNA in the retina ($n$ = 9 cases/sample). mRNA levels are normalized by 18s RNA levels and by levels in normal retina. (*D*) Ocm immunostaining in macrophages. Top: Ocm (green) is concentrated within vesicles of ED-1$^+$ (red) macrophages 3 days after lens injury (LI). Bottom: Control stained with primary antibody preadsorbed with recombinant Ocm. (Scale bar, 10 $\mu$m.) (*E–H*) Western blots of Ocm in normal controls (D0) or day 1 (D1) or 3 (D3) after LI or Zymosan injections (Zy). (*E*) Cells sedimented from the A/V. (*F*) Ocm in the neural retina. Blots were also probed with antibodies to $\alpha$-tubulin ($\alpha$-Tub) to show protein loading. (*G*) Ocm in A/V soluble fraction. ''Mix:'' comigration of recombinant Ocm (4 ng) and A/V sample 1 day after Zy. (*H*) Ocm in vitreous pellet (VP) or retina (Ret) of Sprague-Dawley (SD) rats. Protein from normal lens (15 $\mu$g) was also analyzed. Samples from pooled vitreous pellets and soluble fractions represent quantities present in 1/3 of one eye; retina samples represent protein in 3% of one retina. All blots include recombinant Ocm (4 ng). Western blots to the right of the MW calibrations show key samples probed with the primary antibody omitted or with normal mouse IgG.

(equivalent to 3% of one retina), and the known wet weight of the retina (15–23 mg) (22), the overall concentration of Ocm in the retina 1–3 days after inducing inflammation exceeded 100 nM, which is many times the established $ED_{50}$ (3.8 nM) (17). A soluble pool of Ocm appeared in the A/V supernatant after inducing inflammation (Fig. 1*G*). The slight disparity in migration positions between Ocm in the samples and recombinant Ocm appears to be due to differences in sample composition, and the two co-migrated when combined (Fig. 1*G*). Two strains of rats, including the one used by the lab that failed to detect Ocm (21), showed similar increases in Ocm levels after LI or Zy (Fig. 1*H*). The Ocm band was eliminated when the primary antibody was omitted (Fig. 1 *E*, *F*, and *H*) or when normal mouse IgG was substituted for the primary antibody (Fig. 1*G*).

**Ocm-Blocking Reagents.** We developed two reagents to interfere with the functions of Ocm, a peptide antagonist and a blocking antibody. Ocm binds to RGCs through a domain in its N terminus (17). For the purposes of this study, we narrowed the binding region further to develop a blocking reagent. P1, a peptide representing the N-terminal 24 amino acids of Ocm, bound to RGCs in the low nM concentration range (Fig. 2*A*) and, when present in a molar excess, prevented Ocm from binding (Fig. 2*A*). P3, a peptide representing the next 24 amino acids of Ocm, did not bind to RGCs nor visibly inhibit Ocm binding. In retinal cell cultures, P1 inhibited Ocm (16 nM) from inducing outgrowth in RGCs when present at concentrations at least 5-fold higher than that of Ocm ($P < 0.001$). However, even at high concentrations, P1 did not affect the more modest growth stimulated by CNTF, mannose and forskolin (Fig. 2*B*). The control peptide P3 had no effect in culture when used at 100× the concentration of Ocm, although it showed some effects at higher concentrations. P$\alpha$, another control peptide based on a region of $\alpha$-parvalbumin with 67% similarity to P1, partially inhibited axon growth at high concentrations, but still allowed for a substantial amount of growth (Fig. 2*B*). Neither P1 or P3 affected RGC survival (Fig. S1*A*).

The second reagent tested was an affinity-purified antibody that we generated to the presumed receptor-binding domain of Ocm. This antibody reacted with a single band when tested on Western blots containing soluble proteins of the macrophage cell line from which we initially isolated Ocm (NR8383: American Type Tissue Culture) (Fig. 2*C*). When tested in retinal cultures, the affinity-purified antibody blocked the axon-promoting effects of Ocm, but did not affect the more modest growth induced by CNTF, mannose and forskolin (Fig. 2*D*). Thus, this reagent can be considered as a neutralizing antibody for the next phase of this study. A control rabbit IgG had no effect on Ocm-induced growth (Fig. 2*D*), and neither antibody altered RGC survival in culture (Fig. S1*B*).

**Effect of Blocking Reagents In Vivo.** Both blocking reagents strongly suppressed inflammation-induced optic nerve regeneration. When injected into the vitreous (5 $\mu$L, 2.3 $\mu$g/$\mu$L), P1 decreased inflammation-induced regeneration by 68% ($P < 0.001$) (Fig. 3 *A* and *B*). P3 was inactive at the same concentration, as was P$\alpha$. Like the P1 peptide, the Ocm N-terminal antibody (5 $\mu$L, 1.5 $\mu$g/$\mu$L) suppressed inflammation-induced regeneration by 70% ($P < 0.001$). The control IgG had no effect (Fig. 3 *C* and *D*).

Besides stimulating axon regeneration, intraocular inflammation increases RGCs' ability to survive axon damage (7, 8). However, at the concentrations used to suppress regeneration, neither P1 nor the anti-Ocm antibody diminished RGC survival (Fig. S1*C*).

**Ocm Mediates Inflammation-Induced Regeneration in Other Species and Models.** LI and Zy enhance the ability of RGCs to regenerate axons through a peripheral nerve graft (8, 16). This effect was suppressed with P1 but not by a control peptide (Fig. S2). We also examined the role of Ocm in mice. As in dissociated cultures of mature rat retinas (17), Ocm induced RGCs from early postnatal mice to regenerate axons in the presence of mannose and forskolin (Fig. 4 *A* and *B*). In adult mice, P1 nearly eliminated optic nerve regeneration induced by intraocular inflammation (Fig. 4 *C* and *D*).

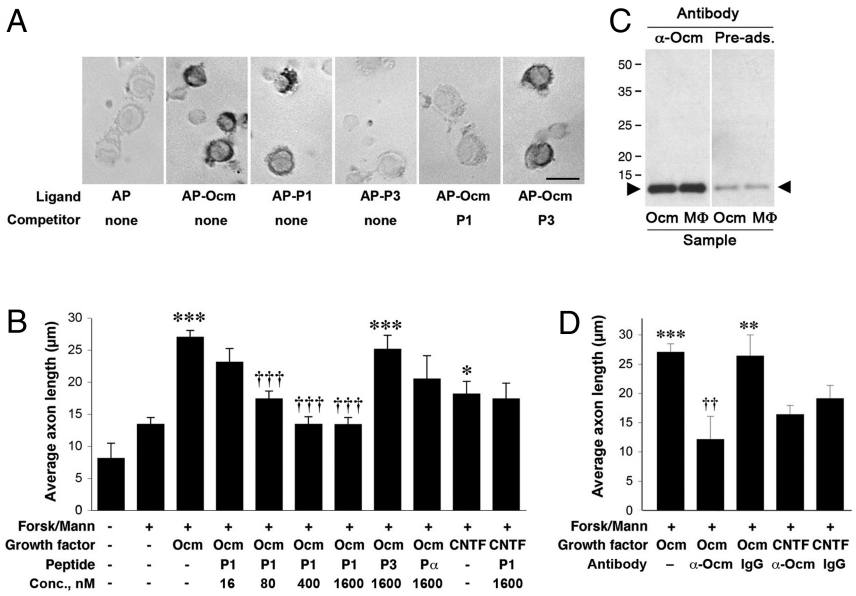


**Fig. 2.** Ocm-blocking reagents. (*A*) P1 is a competitive antagonist of Ocm. An alkaline phosphatase (AP)-Ocm fusion protein, but not AP alone, binds to RGCs in a cAMP-dependent manner (AP-Ocm, AP: 16 nM; forskolin: 15 $\mu$M). An AP fusion with peptide P1, but not with P3, binds to RGCs (both shown at 16 nM). At a 100× molar excess, P1 prevents AP-Ocm from binding. (Scale bar, 20 $\mu$m.) (*B*) P1 blocks the activity of Ocm in culture. Ocm (16 nM) increases outgrowth in the presence of mannose (mann, 250 $\mu$M) and forskolin (forsk, 15 $\mu$M). P1 blocks the response of RGCs to Ocm when present at 5× molar excess or higher, but does not diminish the lesser effect of CNTF (10 nM), even at the highest concentration tested. P3 (1.6 $\mu$M) does not block the effects of Ocm when present at a 100-fold excess, and another control peptide, Pα, partially diminished growth. (*C*) Specificity of the affinity-purified polyclonal anti-Ocm antibody. Left lanes: Western blots of recombinant Ocm and total protein from an immortalized macrophage (MΦ) cell line probed with the affinity-purified polyclonal rabbit IgG generated to the presumed receptor-binding region of Ocm. Right lanes: same samples probed with the antibody after being preadsorbed against recombinant Ocm. Arrowheads show position of Ocm. (*D*) The anti-Ocm antibody suppresses the effect of Ocm in RGCs. The control rabbit IgG has no effect, and neither antibody affects growth stimulated by CNTF, mannose, and forskolin. Negative controls for *D* are the same as shown in *B*. **, ***, Increase different from cells treated with mann + forsk at $P < 0.01$, $P < 0.001$, respectively. ††, †††Decrease different from positive controls at $P < 0.01$, $P < 0.001$, respectively.

## Discussion

The molecular links between inflammation and axon regeneration have been uncertain up to this point. One candidate, Ocm, was previously shown to be secreted by an immortalized macrophage cell line and to induce RGCs to regenerate axons in the presence of appropriate cofactors (17). However, the physiological significance of Ocm has been unclear in the absence of evidence showing that it is actually expressed by inflammatory cells that infiltrate the eye after lens injury or Zymosan, or that it plays an essential role in inflammation-induced regeneration in vivo. In addition, there have been reports that factors other than Ocm can induce regeneration in this system (18–20), that depletion of macrophages or Ocm does not affect regeneration (20, 21), and that Ocm is undetectable in the eye after lens injury (21). Our results contradict these latter findings. We show, first, that Ocm levels increase dramatically in the eye following intraocular inflammation, and second, that Ocm is required for most of the regeneration that occurs irrespective of how inflammation is induced, strain or species studied, or whether regeneration occurs through the optic nerve or through a peripheral nerve graft. These results establish Ocm as an important signal between inflammatory cells and neurons, and as the principal mediator of inflammation-induced regeneration in the optic nerve.

**Ocm Expression.** Quantitative PCR, immunohistochemistry, and Western blotting all demonstrate an association of Ocm with CD68-positive cells that enter the vitreous after injuring the lens. These cells express appreciable levels of Ocm mRNA and contain the protein within intracellular vesicles, consistent with a secretory function seen here and in an earlier study using an immortalized macrophage cell line (17). The retina, in contrast, expresses only low levels of Ocm mRNA but shows high concentrations of the protein within a day of inducing inflammation, presumably derived from the vitreous/aqueous humor. However, we cannot exclude the possibility of other cellular sources. In an earlier study, we reported that retina plus vitreous shows an elevation of Ocm mRNA following inflammation (17), but the present results show that this elevation is due mostly to cells in the vitrous. Ocm expression appears to be under complex regulation. Levels of the mRNA and protein were highest at early stages of inflammation and then declined even as more macrophages continued to enter the eye. In addition, preliminary studies show that peritoneal macrophages express high levels of Ocm mRNA in response to zymosan, but only low levels of the protein. Thus, Ocm expression appears to be regulated in a complex fashion, and is consistent with earlier studies showing that the vitreous environment affects the pattern of macrophage activation and maturation (23).

**Role of Ocm.** Both the peptide competitor P1 and the neutralizing anti-Ocm antibody prevented RGCs from responding to Ocm in culture, and both suppressed inflammation-induced regeneration by approximately 70% in vivo. The P1 peptide binds to RGCs and, when present in molar excess, competitively prevents Ocm from binding. The neutralizing antibody was generated against a highly conserved portion of the Ocm N terminus presumed to be involved in receptor binding, and may sterically prevent Ocm from binding to its receptor. A control IgG did not block the effects of Ocm in culture or in vivo, and the control peptides, while showing some inhibition of Ocm-induced outgrowth, were far less inhibitory than P1 in culture or in vivo. Importantly, neither of the Ocm-blocking reagents diminished outgrowth induced by CNTF, mannose, and forskolin, or affected RGC survival in culture. Thus, the blocking reagents

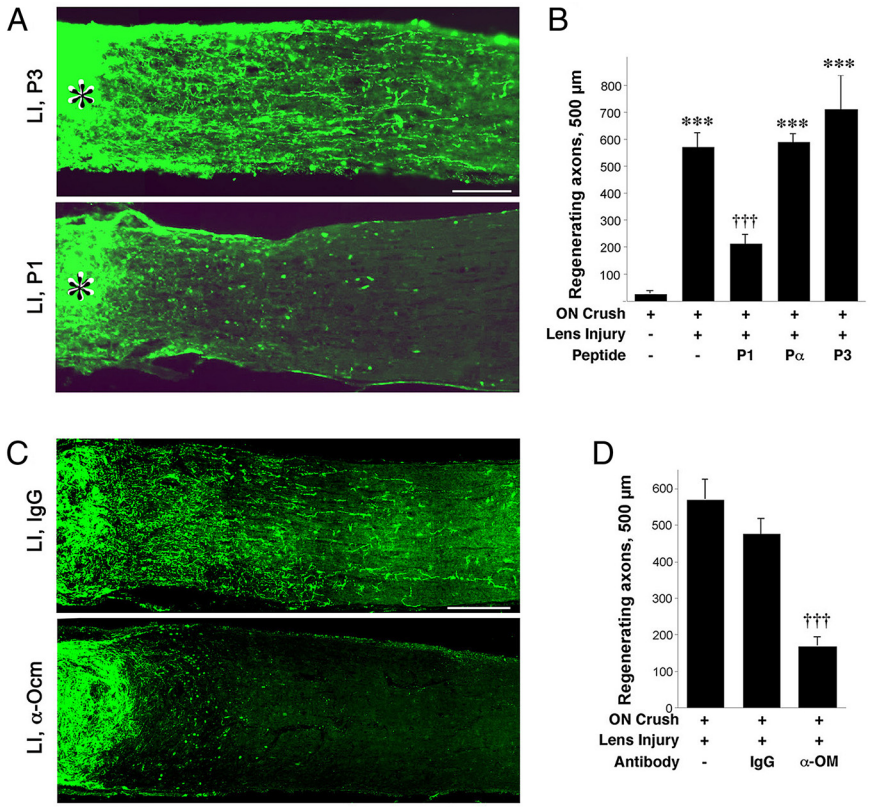


**Fig. 3.** Ocm inhibitors suppress optic nerve regeneration. (*A* and *B*) Effect of peptides on inflammation-induced regeneration in vivo. (*A*) Upper panel: longitudinal section through the optic nerve in a case injected with the P3 control peptide at the time of lens injury. Note the presence of many GAP-43-positive, regenerating axons (green) distal to the injury site (asterisk). Lower panel: regeneration is greatly attenuated in a case injected with the same amount of P1 peptide. (*B*) Quantitation of axon regeneration 2 weeks after surgery and peptide injections. (*C* and *D*) Effects of antibodies on inflammation-induced regeneration in vivo. (*C*) Upper panel: longitudinal section through the optic nerve in a case injected with normal rabbit IgG at the time of lens injury. Note presence of GAP-43-positive axons distal to the injury site. Lower panel: regeneration is greatly attenuated in a case injected with the neutralizing antibody. (*D*) Quantitation of antibody-blocking experiments. Negative controls for *D* are the same as in *B*. ***, Increase different from cells treated with mann + forsk at $P < 0.001$. †††Decrease different from positive controls at $P < 0.001$. (Scale bars in *A* and *C*, 200 $\mu$m.)

appear to interfere specifically with Ocm-induced axon growth per se.

Besides stimulating RGCs to regenerate their axons, intravitreal inflammation attenuates the death of RGCs that normally occurs when axons are injured close to the cell body (7, 8). The Ocm-blocking reagents failed to diminish this effect. These results provide further evidence that the blocking reagents act specifically on Ocm-induced outgrowth, and support our earlier suggestion that factors other than Ocm are responsible for the pro-survival effects of intraocular inflammation (17).

**Other Proposed Molecules and Mechanisms.** Our findings help clarify some of the discrepancies in the literature regarding the molecular basis of inflammation-induced regeneration. One study (21) proposed that macrophages are uninvolved in this phenomenon because a modest depletion of systemic macrophages or local depletion of macrophages in the eye using clodronate liposomes failed to diminish optic nerve regeneration. However, these observations may reflect, respectively, the improved survival of RGCs that occurs by modulating the inflammatory response (8), and the possibility that macrophages may have deposited appreciable amounts of Ocm before ingesting clodronate microspheres in the eye. It is also possible that other immune cells not affected by the microspheres express Ocm. The same group reported that the apparent staining of Ocm on Western blots using a commercial antiserum reflects cross-reactivity with lens protein, but this was shown to not be the case in our studies. In another study, that group reported that the same commercial antiserum did not diminish inflammation-induced regeneration when injected in the eye (20). However, although this antiserum can precipitate Ocm when absorbed onto Protein A beads (17), it has not been shown to be a neutralizing antibody. It was further proposed that CNTF mediates the effect of intraocular inflammation on optic nerve regeneration (20). As we have discussed elsewhere (24), however, CNTF has only weak axon-promoting effects on mature RGCs (present study and refs. 7, 25, 26), and its ability to induce RGCs to regenerate axons in vivo is highly variable across laboratories, and requires concentrations many orders of magnitude above its known $ED_{50}$. Of note, CNTF was recently shown to act in part through an indirect mechanism in inducing RGCs to regenerate axons through a peripheral nerve graft. CNTF was found to stimulate macrophage activation, and its effects on regeneration were suppressed when macrophages were eliminated (27). It remains possible, however, that CNTF could contribute to the effects of intraocular inflammation in enhancing RGC survival (20, 28). The combination of NGF, NT3 and FGF2 has also been reported to promote axon regeneration in the optic nerve (18), although the cellular mechanisms and physiological significance of these findings are presently unknown.

**Conclusion.** The present study establishes Ocm as an important signaling molecule between macrophages and neurons and as the principle mediator of inflammation-induced axon regeneration

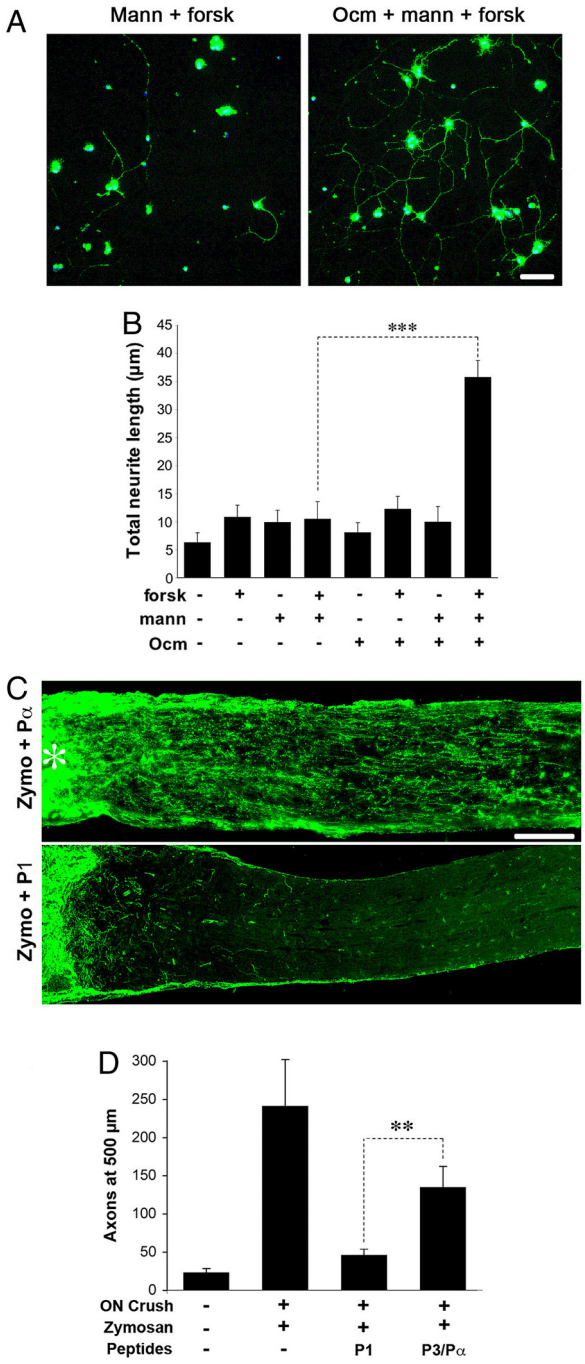


**Fig. 4.** Ocm mediates inflammation-induced regeneration in mice. (*A* and *B*) Ocm enhances axon regeneration in immunopurified postnatal day 3 mouse RGCs in culture. This effect requires the presence of mannose (mann) plus forskolin (forsk). (Scale bar, 45 $\mu$m.) (*B*) Quantitation of cell culture experiments. All conditions were tested in five independent wells and the experiment was repeated three times. ***, Increase relative to forsk + mann significant at $P < 0.001$. (*C*) In vivo, P1 blocks most of the regeneration induced by intravitreal inflammation. Based on preliminary studies showing that, in mice, lens injury had a lesser effect on regeneration than Zymosan, these experiments induced inflammation using Zymosan. (Scale bar, 150 $\mu$m.) (*D*) Quantitation of in vivo studies. Control groups injected with peptides P3 and P$\alpha$ had nearly identical means but showed considerable variability, and were pooled to improve statistical reliability. All groups included four to eight cases except for negative controls ($n = 3$).

in vivo. Additional work will be required to identify the factors that regulate RGC survival after inflammation, determine whether factors other than Ocm contribute to the residual growth seen in the presence of Ocm-blocking reagents in vivo, determine whether Ocm contributes to inflammation-induced axon regeneration in other systems, and discover ways to manipulate the inflammatory response to improve outcome after injury to the optic nerve and other parts of the nervous system.

## Materials and Methods

**Optic Nerve Crush, Lens Injury, and Intraocular Injections.** In vivo work performed at Children's Hospital Boston and at the Chinese University of Hong Kong was approved by the respective Institutional Animal Care and Use Committees. Surgical procedures in rats were similar to those described previously (7, 8) except that we also injected one of the peptides (2.3 $\mu$g/$\mu$L in 5 $\mu$L of 0.1% DMSO-saline, sterilized before use) or antibodies described below at the time of LI. The surgical procedure for adult mice (8–10 weeks of age, strain 129) followed published methods (29) except that, following nerve crush, we removed 3 $\mu$l of fluid from the eye and injected Zymosan (12.5 $\mu$g/$\mu$L in 3 $\mu$L saline: Sigma) with or without one of the peptides. Animals in all groups survived 14 days.

**Ocm-Blocking Reagents.** The Ocm-N terminus includes a sequence that binds to a high-affinity receptor on RGCs but does not stimulate axon outgrowth (17). Peptides representing the first and second 24 amino acids of the N terminus (P1 and P3, respectively) and one covering the central 24 amino acids (P2) were synthesized by Genscript Corp. with a purity >90%. In culture, P1 blocked Ocm-induced axon growth whereas P3 did not. P2 gave variable results and was not used any further. A second control peptide, P$\alpha$, represents an N-terminal region of $\alpha$-parvalbumin with 50% identity (67% similarity) to P1. A third control peptide, P4, was used in the peripheral nerve graft studies, and represents a sequence within the C terminus of Ocm with similar hydrophobicity as P1.

We also used the putative binding region of Ocm as an immunogen to generate a polyclonal anti-Ocm antibody in rabbits (Open Biosystems). Anti-Ocm IgGs were affinity-purified using the immunogenic peptide and tested for specificity and sensitivity on Western blots (Fig. 2*C*). While this antibody proved effective for immunostaining, a previously described monoclonal antibody (gift of Michael Henzl, Univ. Missouri, Columbia MO) proved to be superior for detecting low nanogram quantities of Ocm on Western blots (Fig. 1 *E–H*).

**Evaluating Optic Nerve Regeneration and RGC Survival.** Regenerating axons were visualized in longitudinal sections of the optic nerve by GAP-43 immunostaining, and were quantified in 8–16 sections per case as described (7). RGC survival was evaluated in multiple fields of flat-mounted whole retinas immunostained to visualize $\beta$III-tubulin (1:500, Abcam) as described (30, 31). ED-1$^+$ macrophages were also counted to verify that inflammation had occurred. The significance of between-group differences was determined by ANOVA and Student's *t* tests.

**Peripheral Nerve Graft Model.** The procedures for grafting an autologous section of peripheral nerve to the cut end of the adult optic nerve were the same as described (8, 30) except that rats (Fischer 344) also received an intravitreal injection of one of the peptides at the time of LI. Regeneration was visualized by retrograde transport of Fluorogold (FG, Fluorochrome Inc.) injected into the distal end of the graft 3 days before euthanasia, and was quantified in multiple fields across the retina (8, 30).

**Adult Rat Retinal Cultures.** Mixed cultures containing FG-labeled RGCs were prepared from adult rats as described (8, 17). Each experimental condition was tested in quadruplicate and was evaluated after 3 days by a blinded observer to obtain the average length of the longest axon per cell. Cell survival was defined as the number of FG-positive RGCs per microscope field averaged over 30 fields per well. Values within each experiment are given as the mean ± SEM of four replicate wells. Statistical significance was determined by ANOVA and paired Student's *t*-tests. All experiments were carried out at least three separate times.

**Mouse Retinal Ganglion Cell Cultures.** Mouse RGCs were purified from the retinas of postnatal day 3 C57BL/6 mice by two-stage immunopanning as described ((32) except for the use of anti-CD11b/c antibody-conjugated magnetic beads (BD PharMingen; Dynabead, Invitrogen) to separate out monocytes. Cells that bound to immobilied anti-thy1.2 antibody (Serotec, MCA02R) were released by trypsin and grown in Neurobasal A plus B27 for 3 days (10,000 purified cells per well). Cells were stained with Calcein and Hoescht 33342 and

imaged in 25 fields per well using a Cellomics Kinetiscan. Neurite outgrowth was calculated using the Cellomics Image Analysis Algorithm.

**Analysis of Infiltrative Cells.** Cells that infiltrated the eye were obtained from the aqueous/vitreous fluid (A/V) of Fischer 344 or Sprague-Dawley rats (180–220 g) 1, 3, or 7 days after lens injury or Zymosan injections. For immunostaining and quantitation, one-third of one A/V was cultured on a polyL-lysine-coated coverslip. Adherent cells were fixed after 1 h with 4% paraformaldehyde (10 min, R.T.), incubated with the affinity-purified polyclonal anti-Ocm antibody (1:2,000) and ED1 (1:200 to visualize macrophages) at 4 °C overnight, rinsed, and labeled by the appropriate Alexa Fluor-conjugated secondary antibodies (Invitrogen). As controls, parallel staining was carried out with the primary antibody omitted (negative control) or after absorbing the anti-Ocm antibody with recombinant Ocm protein. The removal of specific anti-Ocm IgGs was verified on Western blots (Fig. 2*C*).

For other analyses, fresh A/V samples were immediately centrifuged to separate cells and the supernatant for Western blots or real-time quantitative RT-PCR.

**Quantitative Reverse Transcription PCR.** Ocm mRNA levels were quantified in retina and in cells sedimented from the A/V. For the latter, we collected three independent samples for each time point, each containing the vitreous humor from 6–16 eyes. RNA was extracted using RNeasy (Qiagen) and was analyzed using TaqMan and SYBR green real-time PCR with a different set of primers from the set used previously (17). Forward: CCAAGACCCGACACCTTTGA; Reverse: GGCTGGCAGACATCTTGGAG. In TaqMan PCR, the probe CACAAAAGTTCTTCCAGACATCGGGCC was used with one-step Master Mix Reagents (Applied Biosystems). The two-step method was also used to confirm the results by using iScript cDNA Synthesis Kit to make cDNA and iQ SYBR Green Supermix for qPCR (both from Bio-Rad). Because the number of macrophages in A/V changes dramatically during inflammation, we report results in terms of absolute mRNA levels, based upon a standard curve of pure Ocm mRNA or cDNA within each analysis. In retinal samples, results were first normalized by 18S rRNA (TaqMan PCR) or GAPDH (SYBR Green qPCR), then normalized by the expression in normal retina.

**Western Blots.** The procedure for preparing samples and detecting Ocm on Western blots was optimized for high sensitivity and low background staining. Details are available upon request. Proteins were separated by SDS/PAGE and transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk for 2 h and then incubated with the monoclonal anti-Ocm antibody (1:200–1:500 in 5% BSA, 4 °C, overnight). After several rinses, an HRP-conjugated secondary antibody (sheep anti-mouse IgG, GE Healthcare Life Science) was applied at 1:2,000 in 2% milk for 1 h. Signals were visualized with ECL-plus. For A/V samples, we loaded a fixed portion of the cell lysates or supernatants (equivalent to 1/3 from one eye per lane) at each time point. For retinal samples, we loaded the equivalent of 3% of the protein in 1 retina. In some cases, filters were also probed with an antibody to $\alpha$-tubulin (1:200,000: Sigma) to visualize overall protein loading. All samples were pooled from three or more cases.

**Ligand Binding Assays.** Plasmids encoding AP-Ocm or AP fusion peptides were expressed in 293T cells and purified with Ni-NTA columns as described (16). Binding assays were carried out in cells that were first treated with forskolin and then lightly fixed with 4% PFA before incubation with AP fusion constructs, with or without competitors present. Positive signals were visualized by the reaction of AP with its substrate NBT/BCIP (17, 33).

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