

# Chemotactic Effect of Ciliary Neurotrophic Factor on Macrophages in Retinal Ganglion Cell Survival and Axonal Regeneration

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**PURPOSE.** To examine whether ciliary neurotrophic factor (CNTF) has a chemotactic effect on macrophages and whether macrophages are involved in CNTF-induced retinal ganglion cell (RGC) survival and axonal regeneration after optic nerve (ON) injury.

**METHODS.** Adult Fischer 344 rats received an autologous peripheral nerve graft onto transected ON for injured axons to grow. CNTF was applied intravitreally. When needed, clodronate liposomes were applied intravitreally or intravenously to deplete macrophages in the eye. A chemotaxis microchamber system was used to examine whether CNTF has a chemotactic effect on macrophages in vitro, whereas immunohistochemistry was used to identify the location of macrophages/microglia in the retina. The effects of CNTF on RGC neurite outgrowth and macrophage/microglia proliferation were tested in retinal explants.

**RESULTS.** Intravitreal CNTF significantly enhanced RGC survival and axonal regeneration as well as the number of macrophages in the eye. Removal of macrophages significantly reduced CNTF-induced RGC survival and axon regeneration. A chemotaxis assay showed a clear chemotactic effect of CNTF on blood-derived but not peritoneal macrophages. Immunohistochemistry revealed that local microglia was located in a region from the nerve fiber layer (NFL) to the inner nuclear layer, whereas blood-derived macrophages were in the NFL. In vitro experiments revealed that CNTF did not enhance neurite outgrowth or macrophage/microglia proliferation in retinal explants.

**CONCLUSIONS.** CNTF is a chemoattractant but not a proliferation enhancer for blood-derived macrophages, and blood-borne macrophages recruited into the eye by CNTF participate in RGC protection. This finding thus adds an important category to the existing understanding of the biological actions of CNTF. (*Invest Ophthalmol Vis Sci.* 2007;48:4257-4266) DOI: 10.1167/iovs.06-0791

Ciliary neurotrophic factor (CNTF) has been known to protect neurons via Janus kinase signal transducer and activators of transcription-3 (JAK-STAT3) signaling pathway.<sup>1-4</sup> We have shown that CNTF is a potent neurotrophic factor for retinal ganglion cell (RGC) survival and axonal regeneration after optic nerve (ON) injury<sup>5,6</sup> or in the presence of ocular hypertension.<sup>7</sup> Gene therapy approaches have shown that a prolonged supply of CNTF in the eye via lentiviral vectors<sup>8</sup> or adenoassociated viral vectors<sup>9,10</sup> also promotes long-term survival of RGCs and regeneration of injured axons in crushed ON or into a peripheral nerve (PN) graft in adult rats. Furthermore, CNTF is neuroprotective of photoreceptors in animal models of retinitis pigmentosa and currently is being tested in a phase I clinical trial for treatment of human retinal degeneration.<sup>11-13</sup>

It is well known that CNTF binds to the CNTF receptor complex to elicit its biological effects.<sup>1,3,14</sup> The receptor complex is composed of extracellular CNTF receptor  $\alpha$  (CNTFR $\alpha$ ) and two transmembrane proteins: gp130 and leukemia inhibitory factor receptor (LIFR) $\beta$ .<sup>1,3,14</sup> Recently, we systematically characterized the signaling transduction underlying CNTF and cAMP elevation-induced RGC survival and axonal regeneration. We showed that after ON injury CNTF/cAMP promotes RGC survival and axonal regeneration via the JAK-STAT3 as well as the PI3K-akt and MAPK-ERK pathways.<sup>15</sup>

Both protective and detrimental effects of macrophages/microglia in neural damage and repair have been shown. Macrophages are often seen to associate with neurodegenerative diseases,<sup>16,17</sup> but these cells can also be neuroprotective.<sup>18</sup> The differential effects of macrophages under different conditions may be accounted for by the different phenotypes of the microglia/macrophages involved.<sup>19</sup> In our previous studies, macrophage activation in the eye by intravitreal injection of zymosan dramatically enhanced RGC survival and axonal regeneration in adult rats.<sup>20</sup> It is known that macrophages produce both beneficial and detrimental molecules, and whether macrophages render neuroprotective or detrimental effects on RGCs depends, at least, on the timing of macrophage activation.<sup>20</sup> Recently, a macrophage-derived factor, oncomodulin, was identified as the most potent molecule known so far in promoting RGC axonal regeneration after ON injury.<sup>21</sup>

Blood-borne macrophages invade the nerve fiber layer (NFL) soon after ON axotomy.<sup>22</sup> In the present study, using both in vivo and in vitro approaches, we investigated whether CNTF is a chemotactic factor that recruits macrophages to the eye and whether these recruited macrophages are involved in CNTF-dependent RGC survival and axonal regeneration. A chemotaxis microchamber system<sup>23,24</sup> was used to verify whether

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CNTF has a chemotactic effect on macrophages and immunohistochemistry, to identify the location of macrophages/microglia in the retina. In addition, a retinal explant model<sup>25,26</sup> was used to examine the effects of CNTF in the absence of blood-derived macrophages.

## METHODS

A total of 63 young adult (10–12 weeks old) Fischer 344 (F344) rats (Vital River, Beijing, China) were used in the study. Forty-eight rats were used for *in vivo* experiments and 15 for immunohistochemistry, macrophage migration assay, and *in vitro* experiments. All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All surgery was performed with the rats under anesthesia of a 1:1 mixture (1.5 mL/kg) of ketamine (100 mg/mL) and xylazine (20 mg/mL).

## Surgical Procedures

The PN-ON surgical procedure used in this study has been described previously<sup>27</sup> and is regularly used in our laboratories.<sup>5,9,10,15,28</sup> Briefly, under anesthesia, the left ON was exposed intraorbitally and transected within the sheath approximately 1.5 mm behind the optic disc. A 1.5-cm piece of autologous peroneal nerve was dissected and sutured with an 11-0 suture onto the proximal stump of the transected ON to provide a permissive environment for injured axons to regrow. The distal part of the PN was secured to connective tissue on the skull.

## Experimental Groups

PN-ON grafted animals were allocated to different experimental groups (Table 1). In the first two groups, saline ( $n = 6$ ) and recombinant rat CNTF ( $n = 6$ ) was injected intravitreally, respectively.<sup>5,15,28</sup> According to the product information (catalog number 450-50; PeptoTech, Rehovot, Israel), the purity of the CNTF used is said to be greater than 98% by SDS-PAGE and HPLC analyses. We confirmed this purity by using an HPLC analysis that showed a single HPLC peak. Because macrophage activation in the eye was seen after CNTF application, we performed additional experiments in which clodronate liposomes were used to remove macrophages in the eye to investigate whether the observed CNTF-induced protection was macrophage dependent. The third to fifth groups received control (PBS) liposomes, clodronate liposomes, and clodronate liposomes+CNTF, respectively ( $n = 5$  each group).

CNTF is known to activate PI3K-akt, MAPK-ERK, and JAK-STAT3 pathways,<sup>15</sup> and macrophage removal was not seen to account for all the CNTF-dependent protections. In the sixth group, we used the MAPK-ERK pathway inhibitor, U0126 (Calbiochem, San Diego, CA) in combination with CNTF to examine whether signal transductions were also involved in CNTF actions ( $n = 5$ ). We were unable to use the inhibitors of the PI3K-akt and JAK-STAT3 pathways, since we found that inhibition of either pathway activated macrophages in the eye (14,710/retina and 12,784/retina by PI3K-akt pathway inhibitors

LY294002 and KY12420, respectively, and 13,496/retina and 16,248/retina by JAK-STAT pathway inhibitors AG490 and Jak inhibitor I, respectively, compared with 1,669/retina in the inhibitor carrier dimethyl sulfoxide [DMSO] group), but application of U0126 was found not to affect the number of macrophages (1987/retina;  $n = 4$ –6 each group).

Single intravitreal injection (3  $\mu$ L) of PBS liposomes or clodronate liposomes was performed immediately after the PN-ON procedure, whereas saline (3  $\mu$ L each injection), CNTF (1.5  $\mu$ g in 3  $\mu$ L each injection), and U0126 (1 mM in 3  $\mu$ L each injection) were injected on days 3, 9, and 15 after PN-ON surgery. For intravitreal injection, the micropipette was deliberately angled to avoid damage to the lens.<sup>29</sup> Clodronate was a gift of Roche Diagnostics GmbH (Mannheim, Germany) and was encapsulated in liposomes, as previously described.<sup>30</sup> Both clodronate and liposomes (if composed of phosphatidylcholine and cholesterol) are not toxic. The clodronate liposomes have been widely used either systemically<sup>31–33</sup> or locally, including in the eye.<sup>34,35</sup> This intravitreal approach was found to be sufficient to remove macrophages in the eye for the 3-week examination period, which indicated that blood-derived macrophages enter the eye only in the early stage after ON injury.

To clarify further whether macrophage degeneration or clodronate liposome degradation in the eye produces side effects that affect RGC viability or whether counteraction of clodronate liposomes with CNTF/U0126 occurs, we applied clodronate liposomes (0.5 mL/100 g body weight) intravenously via tail vein to prevent the invasion of the macrophages and avoid contact of clodronate liposomes with CNTF/U0126. It has been shown that systemic application of clodronate liposomes results in the depletion of blood monocytes after 12 to 18 hours in mice.<sup>33</sup> Other macrophage populations are protected because clodronate liposomes would not cross vascular barriers.<sup>30</sup> Because blood-derived macrophages may only enter the eye in the early stage after ON injury, we applied clodronate liposomes on days 0, 3, and 9 after the PN-ON procedure. Using this intravenous approach, the last three groups received clodronate liposomes ( $n = 4$ ), clodronate liposomes+intravitreal CNTF ( $n = 7$ ) and clodronate liposomes+CNTF+U0126 ( $n = 3$ ; Table 1).

## Retrograde Labeling of Regenerating RGCs

For retrograde labeling of axon-regenerating RGCs, 0.2  $\mu$ L of 4% fluorogold (FluoroGold; Fluorochrome Inc., Denver, CO) was slowly injected into the distal end of the PN graft. The animals were maintained for another 3 days to maximize the retrograde transport of the dye. After euthanization, the rats were perfused and the retinas postfixed with 4% paraformaldehyde for 45 minutes. The orientation of the retinas was marked during retinal dissection.<sup>36</sup> To determine the total number of gold-labeled RGCs in each retina, the number of gold-labeled RGCs in each field (0.25  $\times$  0.25 mm<sup>2</sup>) at a fixed distance from one another, in a pattern of grid intersections, was counted throughout the retina. Sixty to 80 fields were sampled per retina.<sup>15,20</sup>

TABLE 1. Experimental Groups, Number of Animals Used and the Results

PN-ON Plus	Rats (n)	Surviving RGCs/Retina	Regenerating RGCs/Retina	Macrophages/Retina
Saline (3 $\mu$ L in eye)	6	11374 $\pm$ 1434	2150 $\pm$ 459	5446 $\pm$ 915
PBS liposomes (3 $\mu$ L in eye)	5	11411 $\pm$ 2894	2049 $\pm$ 340	5512 $\pm$ 1652
Clodronate liposomes (3 $\mu$ L in eye)	5	11027 $\pm$ 2635	1866 $\pm$ 459	818 $\pm$ 135
Clodronate liposomes (IV) +saline (in eye)	4	9982 $\pm$ 294	1852 $\pm$ 571	2199 $\pm$ 635
CNTF (1.5 $\mu$ g $\times$ 3 in eye)	6	19069 $\pm$ 3798	7988 $\pm$ 1006	15568 $\pm$ 4890
CNTF+U0126 (3 $\mu$ L at 1 mM $\times$ 3 in eye)	5	13756 $\pm$ 543	3506 $\pm$ 1350	16097 $\pm$ 738
Clodronate liposomes (3 $\mu$ L in eye) + CNTF (in eye)	5	14448 $\pm$ 540	3464 $\pm$ 687	7528 $\pm$ 1609
Clodronate liposomes (IV) +CNTF (in eye)	7	16392 $\pm$ 3513	2598 $\pm$ 1196	2863 $\pm$ 1883
Clodronate liposomes (IV) +CNTF/U0126 (in eye)	3	13107 $\pm$ 902	3390 $\pm$ 940	2082 $\pm$ 463

When necessary, clodronate liposomes were used to deplete macrophages in the eye. Liposomes containing PBS were also used as the control. For statistical analysis, see Figure 2 and the text. Data are expressed as the mean  $\pm$  SD.

The average density of FG-labeled RGCs per field was determined and the total number obtained by multiplying this result by the retinal area.

### Immunohistochemical Staining of Viable RGCs and Macrophages

After the number of FG-labeled RGCs was counted, the retinas were carefully brushed off the slides and used for immunostaining of viable RGCs and macrophages. The retinas were thoroughly washed with PBS and blocked with 10% normal goat serum (NGS) and 0.2% Triton for 1 hour. Then, halves of the retinas from a similar region were immunostained overnight at 4°C with a TUJ1 antibody (1:400, anti- $\beta$ III tubulin: BabCO, Richmond, CA) that is found to label specifically the adult RGCs in the eye.<sup>20,28,37</sup> The other halves were immunostained with ED1 (1:400; Serotec, Oxford, UK), which labels macrophages.<sup>20</sup> The retinas were rinsed with PBS and then incubated with conjugated FITC (1:400; Sigma-Aldrich) or cy3 (1:400; Jackson ImmunoResearch, West Grove, PA) secondary antibody overnight at 4°C. After three washes at 5 minutes each, the retinas were mounted with antifading mounting medium (Dako Corp., Carpinteria, CA) and examined by fluorescence microscope. Both  $\beta$ III tubulin positive (4) RGCs and ED1<sup>+</sup> macrophages were counted in the same way as for FG-labeled RGCs.

Though blood-borne macrophages are known to invade the NFL of the retina after ON axotomy, local resident microglia can also be activated by ON injury and become ED1<sup>+</sup>.<sup>22</sup> To investigate the location and morphology of macrophages/microglia in the retina, we performed immunohistochemistry using ED1 and Iba1 antibody that labels both microglia and macrophages in cryosections of saline- and CNTF-treated retinas ( $n = 2$  for each condition). Procedures for ON axotomy and intravitreal applications of saline or CNTF were the same as earlier, but retinas were obtained 2 days after intravitreal saline or CNTF application. Cryosections of the whole eyeballs were cut at 16- $\mu$ m thickness in nasotemporal orientation after postfixation for 2 hours and cryoprotection in 30% sucrose overnight. The retinal sections were thoroughly washed with PBS and blocked with 10% normal goat serum (NGS) and 0.2% Triton for 1 hour. The sections were then immunoreacted with ED1 or Iba1 antibody (1:1000; Wako Pure Chemical Industries, Osaka, Japan) overnight at 4°C. Cy3 (1:400) was used as secondary antibody for 1 hour at room temperature. To identify the location of positive staining in the retina, we added DAPI (1:2000 4',6'-diamino-2-phenylindole; Sigma-Aldrich) at the same time as the secondary antibody, to stain the nuclei of all cells, thus revealing cellular layers of the retina.

### Macrophage Migration Assay

The effect of CNTF on macrophage migration was tested on both blood and peritoneal macrophages. These macrophages were derived from six rats. Macrophage migration was evaluated by using a standard chemotaxis microchamber system (Neuroprobe, Cabin John, MD), as previously described.<sup>23,24</sup> For isolation of blood monocytes, blood was drawn into heparinized syringes from rat heart and was carefully placed on top of a single-density gradient (Percoll, 1.077 g/mL; GE Healthcare, Piscataway, NJ). The content was centrifuged at 400g at 25°C for 30 minutes. The layer of blood mononuclear cells (BMNC) was collected, resuspended in HBSS, and centrifuged twice at 300g for 5 minutes. An amount of  $1 \times 10^7$  BMNC in 5 mL RPMI 1640/10% BSA and penicillin-streptomycin were seeded onto each 6  $\times$  6-cm dish and incubated at 37°C for 1 hour to allow adherence of macrophages to the surface of the dish. The dishes were rinsed three times with cold HBSS to discard the nonadherent cells. Adherent cells were collected from the dish by incubation at 4°C with cell dissociation buffer for 30 minutes. After they were centrifuged for 5 minutes, the cells were collected and resuspended in RPMI 1640 containing 0.1% BSA. For isolation of peritoneal macrophages, the rat peritoneal cavity was filled with HBSS and 5 minutes later peritoneal cavity fluid was collected. After centrifuge for 5 minutes, peritoneal macrophages were washed and the cell pellet was resuspended in RPMI 1640 containing 0.1% BSA.

Both blood and peritoneal macrophages were adjusted to a concentration of  $2 \times 10^5$  cells/mL.

For chemotaxis assay, 25  $\mu$ L of various concentrations of CNTF in RPMI 1640 containing 0.1% BSA were placed into the lower wells of the chemotaxis chamber. Cells (50  $\mu$ L of  $2 \times 10^5$ /mL) were placed in the upper wells of the chamber, which were separated from the bottom wells by a polycarbonate filter (8  $\mu$ m pore size; NeuroProbe). The chamber was incubated at 37°C for 100 minutes. Afterward, cells on the upper surface of the filters were scraped off, and the cells in the filters were fixed with 4% paraformaldehyde and then immunostained with ED1 (1:400) followed by cy3 (1:400). The number of ED1<sup>+</sup> cells in the membrane for each well was counted under a fluorescence microscope. The experiment was repeated three times. Data for the chemotaxis assay are presented as the chemotactic index, which is defined as the number of cells that migrated in the presence of CNTF divided by the number of macrophages that migrated in the presence of medium alone (control).<sup>24</sup>

### Retinal Explant

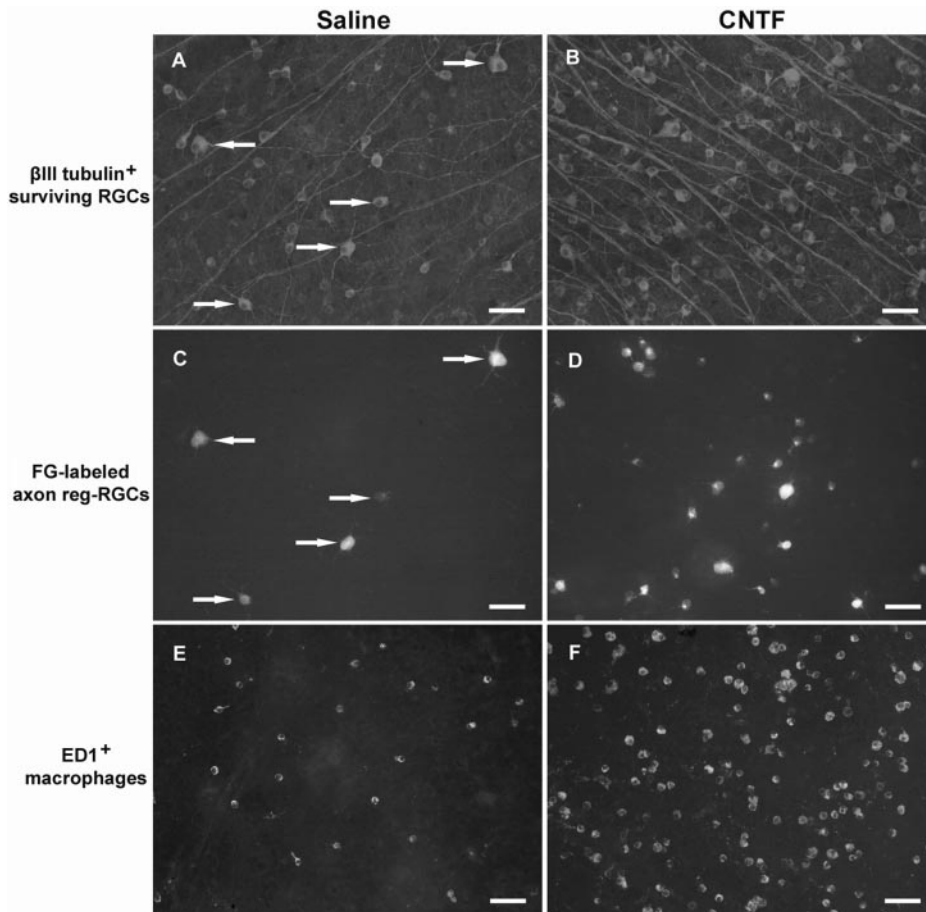
Five rats were used in this experiment. For further investigation of the effect of CNTF on RGC neurite outgrowth when blood-borne macrophages were not available, retinal explants were cultured and treated with CNTF. A culture plate (24 wells) was firstly coated with 300  $\mu$ L poly-lysine (200  $\mu$ g/mL)/well, then with 300  $\mu$ L laminin (20  $\mu$ g/mL in HBSS). In our preliminary studies, retinas without pre-ON crush were found to have low neurite outgrowth ability. This was consistent with a previous report.<sup>38</sup> Thus, a preconditioning ON crush injury was performed, and the retinas were dissected in HBSS 7 days after the ON crush.<sup>25,26,38</sup> After the retinas were mounted onto nitrocellulose filter paper, RGC layer up, each retina was cut into eight pieces, and each piece was cultured in one well in neurobasal A+B27 medium supplemented with glutamine and penicillin/streptomycin. Retinal explants were cultured for 7 days. After fixation in 4% paraformaldehyde for 2 hours, neurites were immunolabeled with TUJ1 antibody (1:400) and macrophages with ED1 (1:400). Five of the longest neurites were measured for each retinal explant, and an average value was obtained and presented as average neurite length in this study. Because neurites tended to regrow in close association with migrating cells (see Fig. 3), it was difficult to determine precisely the number of regrowing neurites in the close vicinity of the explants where numerous migrating cells were present. We counted the number of growing neurites just outside the mass of the migrating cells. All ED1<sup>+</sup> macrophages were counted for each well.

### BrdU Labeling

BrdU (Roche Diagnostics), with or without replenishment of CNTF, was added to the culture medium (final concentration, 10  $\mu$ M) 48 and 4 hours before fixation at culture day 7. Retinal explants were fixed with 4% paraformaldehyde and washed in PBS (pH 7.4) with Triton (three times for 5 minutes). The explants were incubated in HCl (1 N) for 10 minutes at 4°C, followed by HCl (2 N) incubation for 10 minutes at room temperature and then 20 minutes at 37°C to break up the DNA structure of the cells. Samples were washed and then blocked with PBS+1% Triton+10% NGS for 1 hour before incubation overnight with sheep anti-BrdU (1:400; Fitzgerald Industries International, Concord, MA) and mouse ED1 (1:400) antibody for double labeling. After washes (three times, 5 minutes each), the samples were reacted with anti-sheep Alexa Fluor 488 (1:400; Invitrogen-Molecular Probes, Eugene, OR) and anti-mouse cy3 (1:400) secondary antibodies. A field was randomly selected, and the number of ED1<sup>+</sup> and BrdU<sup>+</sup> cells was counted. In each dish, approximately 100 to 200 ED1<sup>+</sup> cells were counted, and the proportion of BrdU<sup>+</sup> proliferating macrophages among them was calculated.

### Statistical Analysis

Data from different groups were statistically analyzed by using one-way analysis of variance (ANOVA) followed by the Bonferroni test,<sup>39</sup> which compares mean values among all groups.<sup>9,15,20</sup>



**FIGURE 1.** Fluorescent photomicrographs showing characteristics of  $\beta$ III tubulin<sup>+</sup> surviving (A, B) and FG-labeled axon-regenerating (C, D) RGCs in the same fields of the retinal wholemounts in saline control and CNTF-treated groups. (A, C, arrows): some of the axon-regenerating RGCs. (E, F) ED1<sup>+</sup> macrophages in the saline control and CNTF-treated groups. Scale bars, 50  $\mu$ m.

## RESULTS

### Effect of CNTF on Macrophage Recruitment into the Eye and the Role of Macrophages in CNTF-Induced RGC Survival and Axonal Regeneration In Vivo

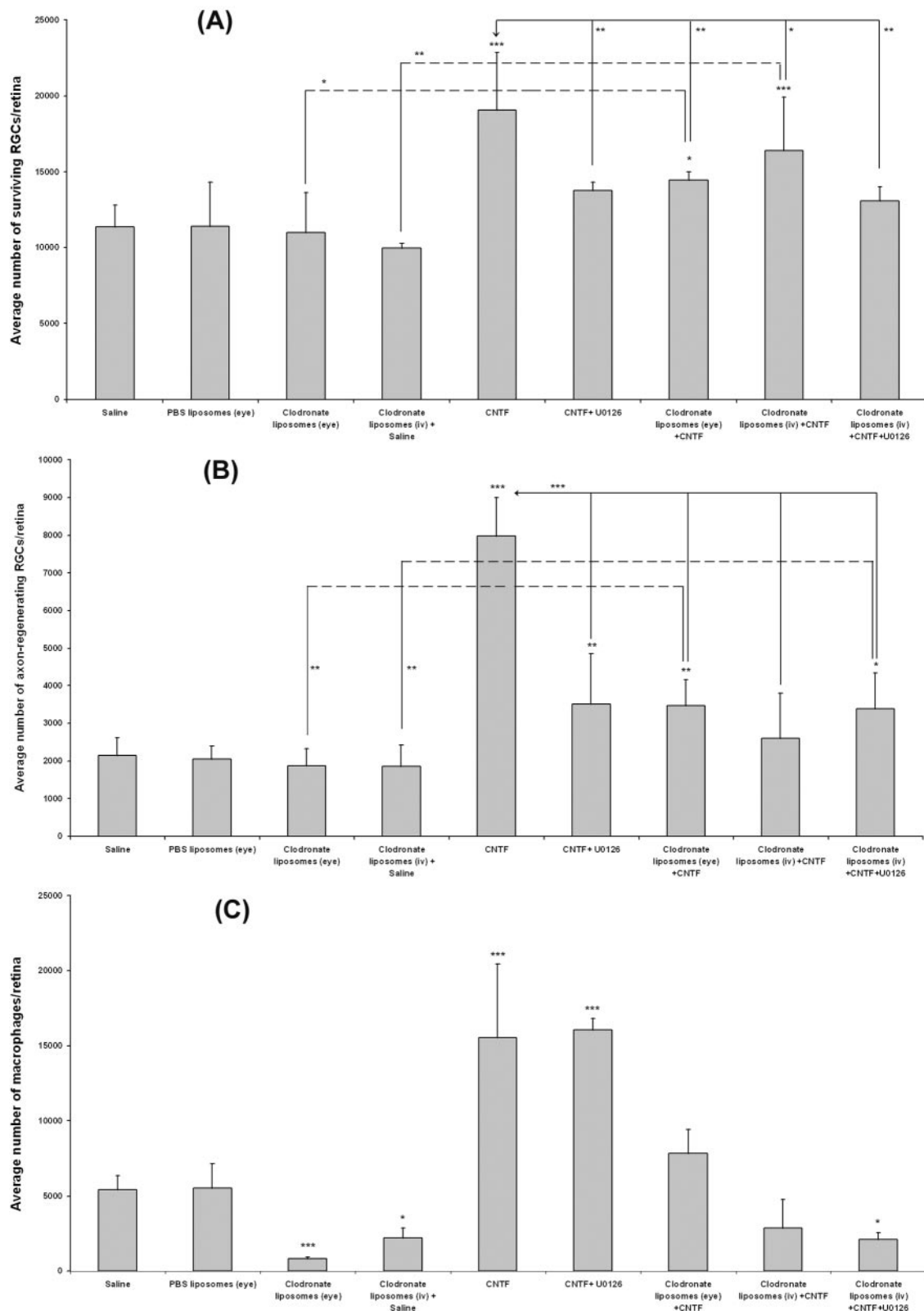
Detailed experimental conditions, the number of rats used, and the results are shown in Table 1. The general appearance of photomicrographs of  $\beta$ III tubulin<sup>+</sup> surviving (Figs. 1A, 1B) and FG-labeled axon regenerating (Figs. 1C, 1D) RGCs in the same field and ED1<sup>+</sup> macrophages (Figs. 1E, 1F) after saline and CNTF treatments are shown in Figure 1. The morphology of invading macrophages appeared to be uniform, in a round activated shape without apparent processes (Figs. 1E, 1F). They appeared to localize on top of the RGCs in the NFL. Note that blood-borne macrophages were shown to invade the NFL in the retina only after ON axotomy.<sup>22</sup> However, immunostaining on retinal cryosections also showed ED1<sup>+</sup> cells in other parts of CNTF-treated retinas (see below).

Three weeks after the PN-ON procedure, the average number ( $\pm$  SD) of  $\beta$ III tubulin<sup>+</sup> surviving and FG-labeled axon regenerating RGCs in the saline treatment group were  $11,374 \pm 1,434$ /retina (Fig. 2A) and  $2,150 \pm 459$ /retina (Fig. 2B), respectively. The average number of ED1<sup>+</sup> macrophages in the retinas of this group was  $5,446 \pm 915$ /retina (Fig. 2C). CNTF significantly ( $P < 0.001$ ) increased RGC survival ( $19,069 \pm 3,798$ /retina; Fig. 2A) and axon regeneration ( $7,988 \pm 1,006$ /retina; Fig. 2B). These results are consistent with our previous observations.<sup>5,6</sup> However, accompanying the enhanced RGC survival and axon regeneration was a significant ( $P < 0.001$ ) increase in the number of macrophages in

the CNTF-treated retinas (Fig. 2C). U0126, previously seen not to influence the number of macrophages in the eye, significantly reduced CNTF-induced RGC survival ( $13,756 \pm 543$ /retina;  $P < 0.05$ ; Fig. 2A) and axonal regeneration ( $3,506 \pm 1,350$ /retina;  $P < 0.001$ ; Fig. 2B), suggesting the involvement of the MAPK pathway in the CNTF-elicited protection.

Single intravitreal application of PBS liposomes or clodronate liposomes did not affect RGC survival and axonal regeneration (Figs. 2A, 2B). However, whereas application of PBS liposomes did not affect the number of macrophages in the eye, clodronate liposomes significantly reduced the number of macrophages to a very low level, indicating the effectiveness of clodronate liposomes in depleting macrophages in the eye for the entire examination period of 3 weeks (Fig. 2C). In fact, the effectiveness of clodronate liposomes in removing macrophages in the eye was seen for 4 weeks after single intravitreal injection at the time of ON injury ( $491 \pm 107$ /retina,  $n = 2$ ). It is likely that blood-borne macrophages entered the eye only in the early stage after ON injury, a proposition supported by a study showing that blood-borne macrophages started to invade the NFL in the retina 5 days after ON axotomy and reached a peak at 7 days.<sup>22</sup> Similar to the intravitreal clodronate liposome group, CNTF-recruited macrophages were also depleted ( $P < 0.001$ ) by the intravitreal clodronate liposome application (Fig. 2C). Of importance, accompanying the absence of recruited macrophages, CNTF-induced RGC survival ( $14,448 \pm 540$ /retina;  $P < 0.05$ ; Fig. 2A) and axonal regeneration ( $3,464 \pm 687$ /retina;  $P < 0.001$ ; Fig. 2B) were significantly reduced.

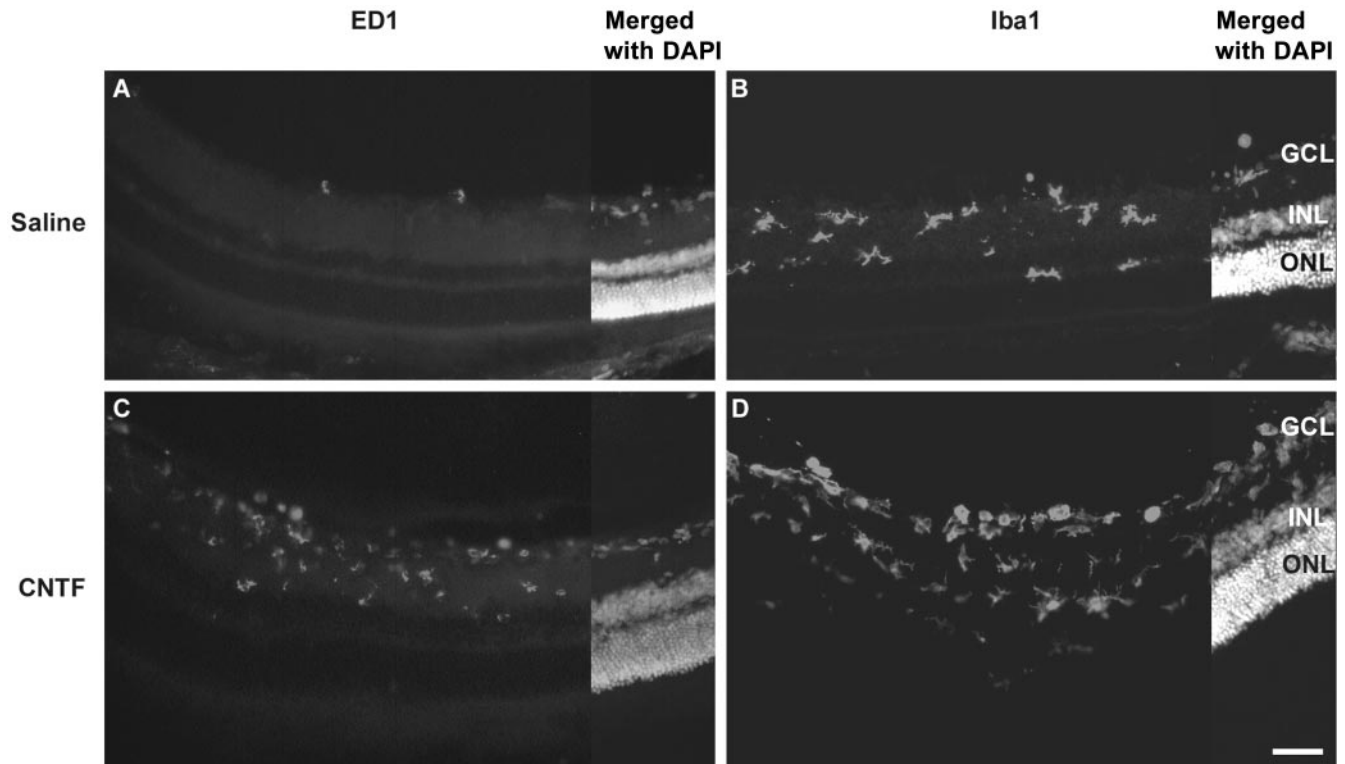
Similar to what were observed after intravitreal application, systemic applications of clodronate liposomes also did not affect RGC survival and axonal regeneration (Figs. 2A, 2B), but



**FIGURE 2.** Average number of  $\beta$ III tubulin<sup>+</sup> surviving RGCs (A), FG-labeled axon-regenerating RGCs (B), and ED1<sup>+</sup> macrophages (C) in various experimental conditions 3 weeks after the PN-ON procedure. Statistical analysis was made against the saline group unless otherwise specified. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , Bonferroni test. Error bars, SD.

substantially ( $P < 0.05$ ) reduced the number of macrophages in the eye (Fig. 2C). The similar results between different clodronate liposome application paradigms indicate that deg-

radation of clodronate liposomes in the eye does not influence RGC survival and axonal regeneration. In the absence of CNTF-recruited macrophages in the eye after intravenous application



**FIGURE 3.** Photomicrographs of eyeball cryosections showing the location of ED1<sup>+</sup> (left column) and Iba1<sup>+</sup> (right column) cells in saline (A, B) and CNTF-treated (C, D) eyes. In each image shown, the right part of the immunostaining image was merged with DAPI staining of the same area, to reveal the cellular layers of the retinas. There were few ED1<sup>+</sup> cells in saline-treated retinas but numerous ED1<sup>+</sup> cells were seen in CNTF-treated ones, mostly in ramified form with processes in the IPL and INL (probably local microglia). In contrast, there were numerous Iba1<sup>+</sup> cells with microglial characteristics (ramified with processes) in saline-treated eyes but more in CNTF-treated eyes. ED1<sup>+</sup> and Iba1<sup>+</sup> cells with processes (probably local microglia) were primarily located in the IPL and INL (B–D), whereas the round shape of the activated form of macrophages without processes (probably blood-borne) was mainly seen in the NFL, more in the temporal area where the CNTF injection was performed (C, nasal retina; D, temporal retina; also see Fig. 1F). Scale bar, 50  $\mu$ m.

of clodronate liposomes, CNTF-induced RGC survival ( $16,392 \pm 3,513$ /retina;  $P < 0.05$ ; Fig. 2A) and axonal regeneration ( $2,598 \pm 1,196$ /retina;  $P < 0.001$ ; Fig. 2B) were again significantly reduced. No significant difference in RGC survival and axonal regeneration was seen between the two clodronate liposome approaches with combined CNTF. Thus, the demise of macrophages did not have a significant influence on RGC survival and axonal regeneration under this condition. Because clodronate liposomes do not cross vascular barriers and other macrophage populations are thus protected,<sup>30</sup> these results confirm the involvement of recruited macrophages from blood circulation in CNTF-dependent protection. Intravitreal CNTF+U0126 treatment combined with intravenous clodronate liposome application yielded similar outcomes in RGC survival and axonal regeneration as in the CNTF+U0126 group without clodronate liposome application (Figs. 2A, 2B). U0126 reduced CNTF-dependent protection in the presence of macrophages (Fig. 2, columns 5 versus 6), but this reduction disappeared after macrophage removal (Fig. 2, columns 6 and 9). These results indicate that the macrophage protection may be MAPK-dependent.

### Morphology and Localization of Microglia/Macrophages in the Retina

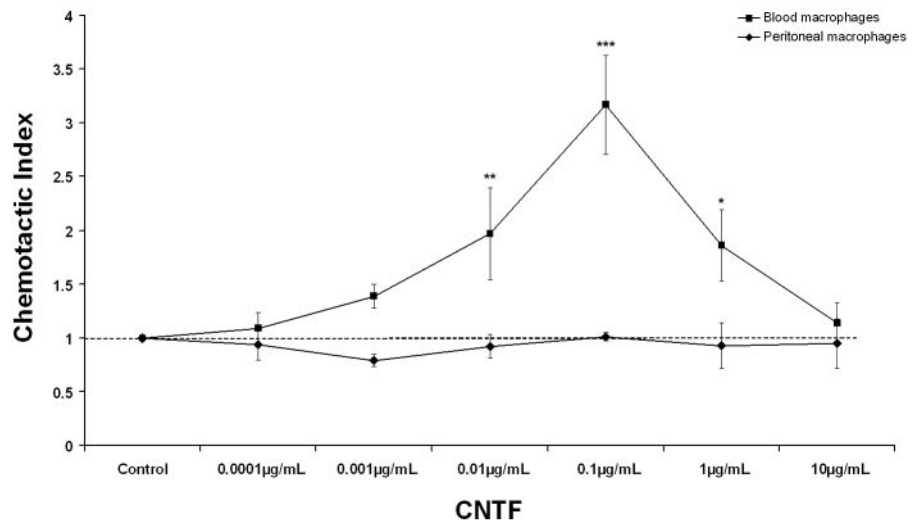
There were few ED1<sup>+</sup> cells in the saline-treated retinas (Fig. 3A). In contrast, numerous ED1<sup>+</sup> cells were seen in CNTF-treated retinas, and they were primarily located in the inner part (NFL-INL; Fig. 3C). Immunohistochemistry using Iba1 antibody revealed numerous cells with strong positive staining in

the inner plexiform layer (IPL) and INL in saline-treated retinas (Fig. 3D). They were of ramified morphology with several processes, characteristic of local microglia. More Iba1<sup>+</sup> microglia were seen after CNTF treatment (Fig. 3D). ED1<sup>+</sup> and Iba1<sup>+</sup> cells with different morphologies were seen at different locations, especially after CNTF treatment (e.g., the round shape of the activated form without processes was seen in the NFL; likely blood-derived macrophages; also see Fig. 1F), especially in the temporal area where CNTF injection was performed, whereas ramified ones with processes (probably local microglia) were evenly distributed in the IPL and INL (Figs. 3C, 3D). These observations indicate that CNTF is influential on both local microglia and blood-borne macrophages. Note that the location of CNTF-recruited ED1<sup>+</sup> cells in the retina in this study was the same as zymosan-activated ED1<sup>+</sup> cells in our previous study.<sup>20</sup>

### Macrophage Migration Assay

Standard chemotaxis microchamber apparatus was used to examine whether CNTF had a chemotactic effect on macrophages in vitro. We found that CNTF exhibited significant activity from 0.01 to 1  $\mu$ g/mL on blood-borne macrophages and displayed a bell-shaped dose-response curve typical of the chemoattractants<sup>24</sup> in this assay (Fig. 4). Significant differences ( $P < 0.001$ ) were also seen between the 0.01- and 0.1- $\mu$ g/mL groups and between the 0.1- and 1- $\mu$ g/mL groups. Thus, these results support our in vivo observations that CNTF acts as a direct chemotactic factor on blood-borne macrophages. In a surprising finding, CNTF was found not to influence migration

**FIGURE 4.** Chemotaxis of CNTF on ED1<sup>+</sup> blood-borne but not peritoneal monocytes. Monocytes were isolated and cultured for 100 minutes in the wells of the upper chamber with the wells in lower chamber containing various concentrations of CNTF. The two chambers were separated by a membrane. The membranes were fixed and stained for ED1. The number of migrating ED1<sup>+</sup> monocytes in the membrane for each well was counted, and the experiments were repeated three times to obtain means and SD. Data are presented as the chemotactic index, which is defined as the number of cells migrating in the presence of CNTF divided by the number of macrophages migrating in the presence of medium alone (control).<sup>24</sup> Statistical analysis (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , Bonferroni test) was made against the control (dotted line). Significant differences ( $P < 0.001$ ) were also seen between the 0.01- and 0.1- $\mu\text{g}/\text{mL}$  groups and between the 0.1 and 1  $\mu\text{g}/\text{mL}$  groups. Error bars, SDs.

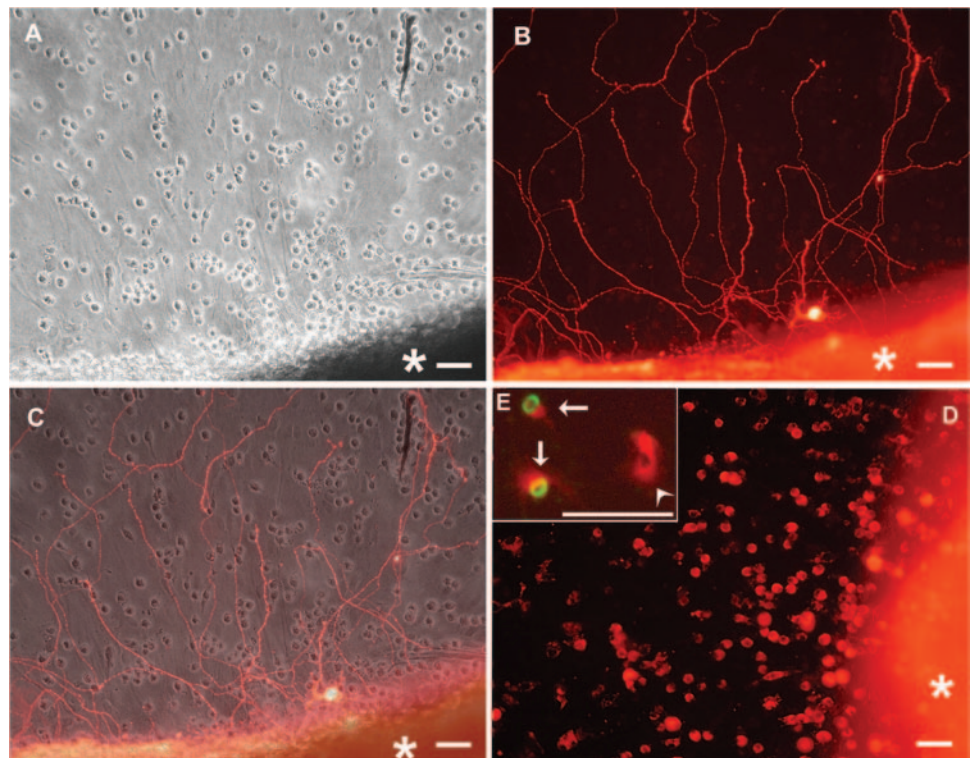


of peritoneal macrophages (Fig. 4). However, the latter observations are consistent with what were seen in a similar assay.<sup>24</sup>

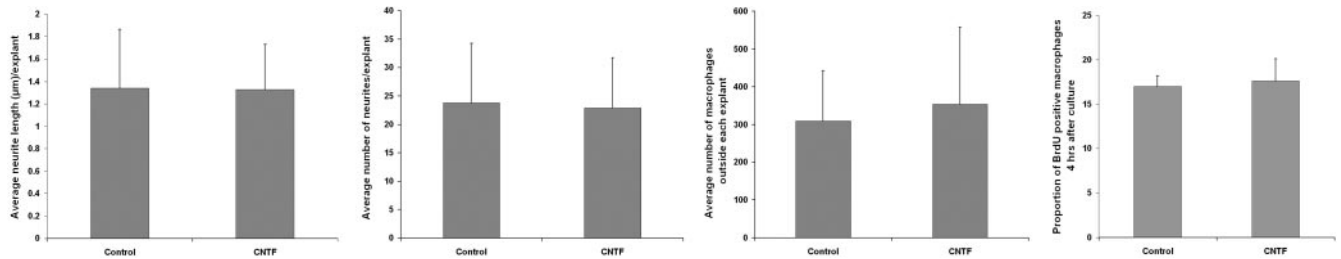
### Effect of CNTF in RGC Neurite Outgrowth in Retinal Explants

The exemplified appearance of migrating cells (Fig. 5A),  $\beta$ III tubulin<sup>+</sup> growing neurites (Fig. 5B) in the same field (Fig. 5C; merged), and ED1<sup>+</sup> macrophages migrating outside the retinal explants (Fig. 5D) are shown in Figure 5. There was no obvious effect of CNTF on either the number or the length of outgrowing neurites in retinal explants (Figs. 6A, 6B). This is accompanied by a lack of effect on the number of macrophages presented outside the explant (Fig. 6C). Note that blood-derived macrophages were not available in this in vitro condition.

These data are consistent with a previous report in which minimal effect on RGC neurite outgrowth was seen in adult retinal explants.<sup>40</sup> To investigate whether CNTF enhanced local microglial proliferation, BrdU experiments were performed. No obvious enhancement in the proportion of BrdU<sup>+</sup> cells among ED1<sup>+</sup> cells was seen in the CNTF group shortly (4 h) after BrdU administration (Fig. 6D). The ability of ED1<sup>+</sup> cells to proliferate was high, as 95% of randomly selected ED1<sup>+</sup> cells ( $n = 215$ ) were also BrdU<sup>+</sup> 48 hours after culture. As macrophages secrete various molecules, it is possible that CNTF acts in concert with certain macrophage-derived factors to enhance RGC axonal regeneration in vivo. In the in vitro situation where macrophage-derived factors are not readily available, the effect of CNTF on neurite outgrowth becomes diminished.



**FIGURE 5.** Photomicrographs of retinal explants showing characteristics of (A) migrating cells and (B)  $\beta$ III tubulin<sup>+</sup> neurites in the same field. (C) Merger of images in (A) and (B). (D) ED1<sup>+</sup> cells outside the retinal explant and (E) ED1<sup>+</sup> cells that are BrdU<sup>+</sup> (arrows) and negative (arrowhead). (\*) Edge of the retinal explant. Cells within the explant were not visible, owing to the filter on top of the explants. The large number of ED1<sup>+</sup> cells outside the explant may result from proliferation. Scale bars, 50  $\mu\text{m}$ .



**FIGURE 6.** Average number (A) and length (B) of  $\beta$ III tubulin<sup>+</sup> neurites, average number of ED1<sup>+</sup> macrophages (C) outside the retinal explants, and proportion of BrdU<sup>+</sup> macrophages (D) under various conditions in retinal explant culture. No statistically significant difference was seen. Note that the proliferative ability of macrophages in culture is high (D). Error bars, SD.

## DISCUSSION

Recently, we showed that CNTF not only activates the JAK-STAT3 pathway but also the MAPK-ERK and PI3K-akt pathways to exert its biological effects.<sup>15</sup> In the present study, we extended the function of CNTF to be a chemoattractant on blood monocytes and recruited them into the eye. We showed that the CNTF-recruited macrophages in the eye in turn helped promote RGC survival and axonal regeneration. Macrophage migration assay using the chemotaxis microchamber system confirms the chemotactic action of CNTF on blood-derived but not peritoneal macrophages. In contrast, despite the large number of macrophages in the eye, CNTF-induced RGC survival and axonal regeneration were significantly reduced by the MAPK-ERK pathway inhibitor U0126, suggesting that other conventional signaling transduction mechanisms were also involved in CNTF-dependent actions in the eye. These mechanisms may achieve RGC protection via indirect action on macrophages/microglia rather than directly on RGCs or indirectly on other cellular populations in the retina, since the U0126-related blockade of CNTF-dependent RGC protection disappeared in the absence of recruited macrophages in the eye.

It is known that macrophages produce various types of cytokines and neurotrophic factors,<sup>41</sup> and whether stimulated macrophages render protective or detrimental effects depends on the timing of macrophage activation.<sup>20</sup> CNTF protection in RGC survival and axonal regeneration after ON injury is probably achieved by the combined action of various molecules in the eye. This notion is partially supported by the lack of CNTF action in neurite outgrowth *in vitro* but enhancement of axonal regeneration *in vivo* in this study.

Blood-borne monocytes have been shown to invade the NFL of the retina after ON transection.<sup>22</sup> Immunohistochemistry in this study showed that CNTF affected the number of both ramified local microglia and blood-derived activated macrophages in the eye. Previously, CNTF was shown not to exert a chemotactic effect on peritoneal macrophages in a similar chemotaxis assay approach, but potentiated CNTFR $\alpha$ -dependent macrophage chemotaxis in a PI3K-akt and MAPK-ERK dependent manner.<sup>42</sup> The present study shows that CNTF alone is sufficient to exert chemotactic action on blood-derived monocytes and recruits these monocytes into the eyes. These results are thus in accord with those observed *in vivo* in this study. Because macrophages express receptors such as gp130 and LIFR $\beta$  for CNTF,<sup>42</sup> the enhanced availability of these receptors provided by CNTF-recruited macrophages would in turn help potentiate CNTF actions on these macrophages.

It is surprising to see that CNTF is a chemoattractant for blood but not peritoneal monocytes, though the latter is consistent with findings in a previous report.<sup>24</sup> Because CNTFR $\alpha$ - or CNTFR $\alpha$ +CNTF-elicited macrophage chemotaxis was inhibited by a neutralizing gp130 antibody,<sup>42</sup> it is possible that the level of gp130 is different between blood and peritoneal mono-

cytes. It is also possible that blood and peritoneal monocytes express different levels of CNTFR $\alpha$  and LIFR $\beta$ , the other two components of the CNTF receptor complex. Downstream of signaling transduction after binding with the receptor complex, CNTF is now known to act via JAK-STAT, PI3K-akt, and MAPK-ERK signaling transduction pathways,<sup>15</sup> to exert its biological effect. The signaling transduction activities of JAK-STAT, PI3K-akt, and MAPK-ERK pathways in blood and peritoneal monocytes may be different, an aspect currently under investigation in this laboratory. All these differences may contribute to the different chemotactic responses of blood and peritoneal macrophages to CNTF. It should be noted that, apart from macrophages, CNTF-mediated signaling transduction may also directly modulate neuronal survival and neurite outgrowth. Because it is known that after binding with CNTFR $\alpha$ , CNTF acts directly on neurons via signaling transduction of JAK-STAT, PI3K-akt, and MAPK-ERK pathways *in vitro*,<sup>1-3,43,44</sup> our *in vivo* results from the clodronate liposomes (intravenously [IV])+CNTF group and the clodronate liposomes (IV)+CNTF+U0126 (in eye) group suggest the same.

There are numerous resident microglia (local macrophages in the central nervous system) in the normal adult retina. Previous studies have demonstrated that a variety of antibodies, such as OX42 and ED1, label microglia and macrophages.<sup>45,46</sup> Recently, it has been shown that resident retinal microglia activate and convert into macrophages after such stimulations as ON injury and retinal ischemia, and that most OX42<sup>+</sup> cells are also ED1<sup>+</sup>.<sup>47,48</sup> Our ED1 immunohistochemical results suggest that CNTF also induces local microglia to become ED1<sup>+</sup>, and the Iba1 results show that positive cells with different morphologies locate at different regions in the retina. Blood-borne macrophages have been shown to invade the retina, but only in the NFL after ON transection.<sup>22</sup> The differences in the number, morphology, and location of Iba1<sup>+</sup> cells after saline and CNTF treatment, coupled with the reduction of ED1<sup>+</sup> macrophages in retinal wholemounts after intravenous application of clodronate liposomes *in vivo*, suggest that the CNTF-recruited macrophages in the eye are probably from the blood circulation and CNTF is influential on both local microglia and blood-borne macrophages. The proliferative ability of ED1<sup>+</sup> cells in culture was high, as 95% of ED1<sup>+</sup> cells were also BrdU<sup>+</sup> after 48 hours in culture. However, CNTF did not enhance proliferation of ED1<sup>+</sup> macrophages in retinal explants.

In conclusion, in the current study intravitreal application of CNTF recruited blood-borne macrophages into the eye, and the recruited-macrophages participated in CNTF-induced RGC survival and axonal regeneration after ON injury. MAPK-ERK may underlie the macrophage-independent protection of CNTF in the eye. The novelty of our findings is that, in addition to known biological actions via signaling pathways,<sup>1-4,43,44</sup> CNTF also exerts neuroprotection via chemotactic action on macro-



phages. This finding thus adds an important aspect to our existing understanding of the biological actions of CNTF.

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