TNF-α-Induced Optic Nerve Degeneration and Nuclear Factor-κB p65

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PURPOSE. To characterize a model of optic nerve axonal degeneration induced by tumor necrosis factor (TNF)-α and to determine the role of nuclear factor (NF)-κB p65 in axonal degeneration.

METHODS. Groups of rats were euthanatized at 1 day, 1 or 2 weeks, or 1 or 2 months after intravitreal injection of TNF-α. Morphometric analyses of neurofilament- or Thy-1-positive cells, retinal ganglion cells (flat preparations stained with cresyl violet or retrograde labeling with a neurotracer), the number of axons, immunostaining for myelin basic protein, and TUNEL assays were performed. Levels of NF-κB p65 protein in retina and optic nerve were determined by Western blot analysis and immunohistochemistry. The effects of antisense oligodeoxynucleotide (AS ODN) against NF-κB p65 and helenalin, an inhibitor of NF-κB p65 activation, on TNF-α-induced optic nerve degeneration were determined by counting the number of axons.

RESULTS. Intravitreal injections of TNF-α induced obvious axonal loss and extensive degeneration of the axons from 2 weeks to 2 months after injection, whereas significant retinal ganglion cell loss was noted only at 2 months after injection. NF-κB p65 was increased in the optic nerve but not in the retina and was found to colocalize with ED-1 and Iba1, markers of microglia. Inhibition of NF-κB p65 with AS ODN or helenalin significantly ameliorated the effects of TNF-α-mediated axonal loss.

CONCLUSIONS. TNF-α causes axonal degeneration with probable delayed loss of retinal ganglion cell bodies. NF-κB p65 may play a pivotal role in axonal degeneration, with the possible involvement of microglial cells. (*Invest Ophthalmol Vis Sci.* 2006;47: 1448–1457) DOI:10.1167/iovs.05-0299

Axonal degeneration in the central nervous system (CNS), as seen in such neurodegenerative diseases as multiple sclerosis[1] and Alzheimer's and Parkinson's diseases,[2] often lead to the death of neuronal cell bodies. Axotomy in the brain such as neurohypophysectomy, is known to lead to retrograde degeneration of the magnocellular neurons.[3] Similarly, axotomy of the optic nerve, which is primarily made of retinal ganglion cell (RGC) axons, leads to apoptotic degeneration of RGCs.[4] Axonal degeneration of RGCs and apoptotic death of the cell bodies are also observed in glaucoma in which elevated intraocular pressure is known to be a major risk factor. Yet, it remains unclear whether RGC death occurs via retrograde degeneration of the optic nerve or in combination with a direct insult to the cell body. Recent studies in Wallerian degeneration slow (Wld[s]) mutant mice demonstrated that axonal degeneration of neurons can be a highly regulated process that is distinct from the apoptotic pathway(s).[5] Overexpression of Bcl-2, an antiapoptosis gene, can prevent neuronal cell death but not axonal degeneration in a genetic mouse model of motor neuron disease.[6] Understanding the mechanism of axonal degeneration in neurodegenerative diseases through the development of an animal model system is crucial in formulating new strategies for the discovery of neuroprotective therapies.

Tumor necrosis factor (TNF)-α, a cytokine that is synthesized and released from astrocytes and microglia in the CNS, has been implicated in several neurodegenerative diseases, such as multiple sclerosis,[7] Alzheimer's disease,[8] human immunodeficiency virus (HIV)-related encephalopathy,[9] and HIV-related optic neuropathy.[10] TNF-α-induced cell death occurs primarily as mediated by TNF-receptor 1 (TNF-R1), which may interact with the adaptor TNFR-associated death domain (TRADD) through its cytoplasmic region.[11] TRADD, in turn, associates with the death domain (DD) of Fas-associated death domain (FADD), thereby initiating the caspase-8 dependent apoptotic pathway.[12] TRADD can also recruit the adaptor protein TNF-R-associated factor 2 (TRAF2) which induces the activation of the nuclear factor (NF)-κB signaling.[13] In contrast, TNF-α may serve a neuroprotective function through its activation of TNF-receptor 2 (TNF-R2),[14] which recruits TRAF2, which in turn induces NF-κB activation.[15] Therefore, TNF-α action in vivo may be complicated depending on the expression of different populations of receptor subtypes.

In the eye, TNF-α-mediated neurotoxicity has been linked to optic nerve degeneration in patients with glaucoma[16-18] and in those with HIV-related optic neuropathy.[10] Upregulation of TNF-R1 was reported in glaucomatous optic nerve heads.[17] In addition, earlier studies demonstrated that intravitreal injection of TNF-α induces axonal degeneration in mouse[19] and rabbit[20] optic nerve. Other studies demonstrated that TNF-α release by glial cells is induced on exposure to simulated ischemia and elevated hydrostatic pressure, resulting in apoptotic RGC death.[21] These findings support the hypothesis that TNF-α may be involved in ocular disease neurodegeneration.

In this study, intravitreal TNF-α induced progressive axonal degeneration with slow retinal neuronal involvement. NF-κB

p65 and microglial cells colocalized in TNF-α-mediated axonal degeneration and inhibition of NF-κB p65 led to axonal protection. Our study may serve as an in vivo model for investigating axonal degeneration.

MATERIALS AND METHODS

Animals

Experiments were performed on 50- to 55-day-old male Lewis albino rats. All studies were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals lived in controlled conditions, with temperature at 23 $\pm$ 1°C, humidity at 55% $\pm$ 5%, and light from 6 AM to 6 PM.

Administration of TNF-α

Intravitreal injection of TNF-α (Sigma-Aldrich, St. Louis, MO) was performed as described previously,[20] with a slight modification. Briefly, rats were anesthetized by intramuscular injections of a mixture of ketamine-xylazine (10 and 4 mg/kg, respectively). A single 2-μL injection of 1 or 10 ng TNF-α in 0.1 M phosphate-buffered saline (PBS, pH 7.40) was administered intravitreally into the right eye of an animal under a microscope, to avoid lens injury. PBS was administered into the contralateral left eye as a control. The rats were euthanatized at 1 day, 1 or 2 weeks, or 1 or 2 months after the injection by intraperitoneal injection of an overdose of pentobarbital sodium followed by enucleation of the eye. For the dose–response/axon number study, 0.1, 1, 10, or 100 ng TNF-α in 0.1 M PBS was administered intravitreally, and eyes with optic nerves were removed 2 weeks after injection.

Western Blot Analysis

Sixty-two rats were used for Western blot analysis, as described previously.[22] Briefly, after day 1 of intravitreal injection, retinas and optic nerves (4 mm length of optic nerve, removed right behind the globe) were collected, homogenized, and centrifuged at 15,000g for 15 minutes at 4°C. One optic nerve preparation included four optic nerves, whereas each retina served as an individual sample. Protein concentrations were determined using the Protein Assay kit (Bio-Rad, Hercules, CA). Protein samples (5 μg per lane for the optic nerve and 10 μg per lane for the retina) were subjected to SDS-PAGE on 10% polyacrylamide gels and transferred to enhanced chemiluminescence (ECL; GE Healthcare, Piscataway, NJ) membranes. The membranes were blocked with Tris-buffered saline (TBS) and 0.1% Tween-20 containing 5% skim milk. The membranes were first reacted with anti-NF-κB p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (GAPDH; Chemicon International, Temecula, CA), both diluted 1:200 in TBS containing 5% skim milk. The membranes then exposed to peroxidase-labeled anti-rabbit IgG antibody (Sigma-Aldrich) diluted 1:5,000 or peroxidase-labeled anti-mouse IgG antibody (Sigma-Aldrich) diluted 1:50,000 in Tween-20 in TBS. Western blots were visualized with a chemiluminescence detection system (ECL Plus Western Blot Detection Reagent; GE Healthcare).

Immunohistochemistry and In Situ TUNEL Assay

Eyes collected at 1 day, 1 or 2 weeks, or 1 or 2 months after intravitreal injection were fixed by immersion in 10% neutral buffered formalin for 24 hours, dehydrated, embedded in paraffin, and sectioned (4 μm thick) through the optic disc. Deparaffinized sections were incubated with 1% bovine serum and then reacted with primary antibodies against NF-κB p65 (1:100; Santa Cruz Biotechnology), ED1 (a marker of microglia/phagocytic cells; 1:100; Serotec, Raleigh, NC), Iba1 (a marker of microglial cells; 1:50; Santa Cruz Biotechnology), vimentin (a marker of glia; 1:20; Chemicon International), myelin basic protein (MBP; 1:200; DakoCytomation Corp., Carpinteria, CA), neurofilament-L

(1:100; DakoCytomation Corp.), or Thy-1 (1:50; Santa Cruz Biotechnology), diluted in 1% bovine serum overnight at 4°C. Sections were then exposed to the following secondary antibodies: FITC-labeled anti-rabbit antibody (1:100), TRITC-labeled anti-mouse antibody (1:100), or TRITC-labeled anti-goat antibody (1:100; all from DakoCytomation Corp.). Immunochemistry of MBP and neurofilament was performed with the diaminobenzidine (DAB) detection method with an anti-rabbit antibody (EnVision System; DakoCytomation Corp). For Thy-1 immunostaining, the samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vectashield with DAPI; Vector Laboratories, Burlingame, CA). Negative controls were performed by replacing the primary antibody with PBS or serum. Specimens from the control groups were included and compared with those treated with TNF-α.

Neurofilament-positive cells in the RGC layer (RGCL) were manually counted in retinal sections containing the whole retina and passing through the optic nerve. Regions within 2 mm (both sides, total 4 mm length) from the edge of optic disc were used for counting. Data from three sections of each eye were averaged for one eye. At least four eyes were examined in each experimental condition.

Thy-1-positive cells in the RGCL were analyzed in the images captured by a fluorescence confocal microscope. Three images of regions at approximately 0.5 to 1.5 mm from the edge of the optic disc were obtained in one side (six images in both sides), and three sections were used for quantification in one eye (18 images in each eye). Data from three sections of each eye were averaged for one eye and four eyes were examined in each experimental condition.

For the in situ TUNEL assays, retinal sections obtained as described in the preceding section were used. Endogenous peroxidase was inactivated by incubating the sections with 0.3% H_2O_2 for 10 minutes at room temperature. The TUNEL assay was performed (ApopTag Peroxidase In Situ Apoptosis Detection kit; Intergen Co., Purchase, NY), with DAB used as the color substrate.

Axon Counting in Optic Nerves

Morphometric analysis of each optic nerve was performed as described previously[20,23] with samples from 53 rats. Eyes were obtained from animals at 1 or 2 weeks and 1 or 2 months after intravitreal injection. Two-millimeter segments of optic nerve were obtained starting at 2 mm behind the globe. These segments of optic nerve were fixed by immersion in Karnovsky's solution for 24 hours at 4°C, processed, and embedded in acrylic resin. Cross-sections (1 μm thick) were cut beginning at 2 mm from the globe and stained with a solution of 1% paraphenylene-diamine (PPD) in absolute methanol.[20] For each section, images at the center and at each quadrant of the periphery (approximately 141.4 μm from the center) were acquired with a light microscope (Axioskop; Carl Zeiss Meditec, Dublin, CA) at 1250×, with a charged-coupled digital camera (7.3 Three shot color; Diagnostic Instruments, Inc.) and associated software (Spot, ver. 4.0.9; Diagnostic Instruments, Inc.). The acquired images were quantified on computer (Aphelion image-processing software; ver. 2.3; ADCIS SA and AAI, Inc.; Caen, France).[23] The number of axons was determined in five distinct areas of 2,713.5 μm^2 each (each quadrant of the periphery in addition to the center; a total area of 13,567.5 μm^2 per eye) from each eye. The number of axons was averaged per eye and expressed as the number per square millimeter. A minimum of four eyes per experimental condition was used for analysis.

Morphometry of Cells in the RGCL

Morphometry of cells in the RGCL on wholemounted flat preparations of retinas was performed as described previously,[24] in samples from 29 rats. Briefly, eyes obtained from animals at 2 weeks or 1 or 2 months after intravitreal injection, were fixed by immersion in 10% neutral buffered formalin for 1 hour. Retinas were separated and flatmounted in a 1% gelatin solution with the RGCL facing upward. These preparations were stained with 1% cresyl violet. The number of RGC-like cells
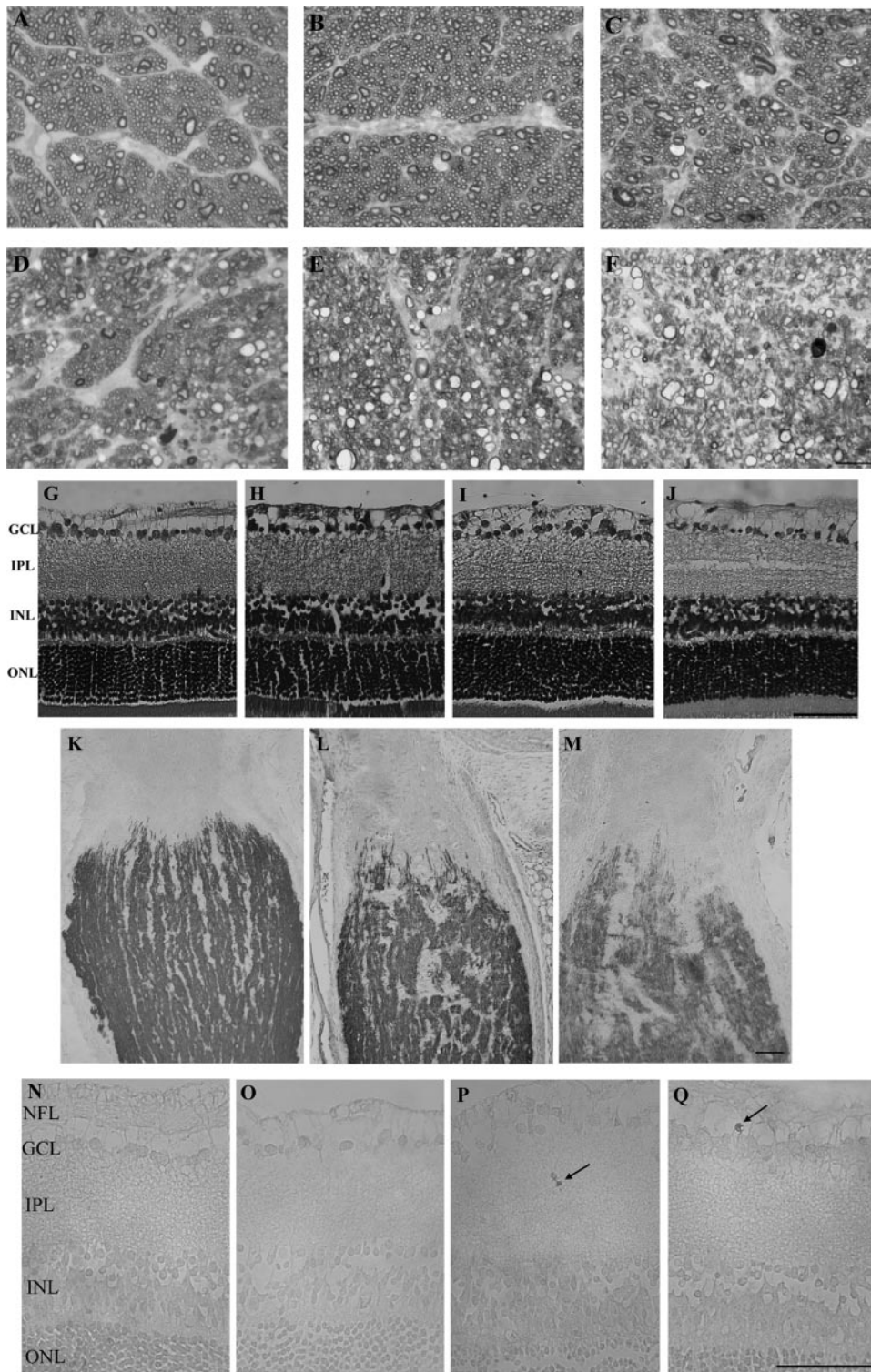
FIGURE 1. Histo- and immunopathology of optic nerves and retinas after TNF-α injection. (**A–F**) Optic nerve morphology: (A) normal; (**B**) 1 week after vehicle injection (unremarkable); (**C–F**) substantial and progressive axonal degeneration at (**C**) 1 week, (**D**) 2 weeks, (**E**) 1 month, and (**F**) 2 months, respectively, after 10 ng TNF-α injection. (**G–J**) Morphology of retinas: All retinal layers were well-preserved in (**G**) normal retina and in retinas (**H**) 1 week, (**I**) 2 weeks, and (**J**) 1 month after 10 ng TNF-α injection. No difference was detectable between normal and PBS-treated eyes during the whole observation period (not shown). (**K–M**) MBP immunoreactivity in the optic nerves. (**K**) Normal optic nerve and optic nerves (**L**) 1 week and (**M**) 2 weeks after TNF-α injection. No difference was detected between normal and PBS-treated eyes (not shown). Note the progressive loss of MBP immunoreactivity in the optic nerve after TNF-α injection. (**N–Q**) In situ TUNEL of retinas: TUNEL-positive cells were undetectable in (**N**) normal and (**O**) PBS-treated retinas. A few positive cells (*arrows*) were observed in the NFL and IPL at (**P**) 1 and (**Q**) 2 weeks after 10 ng TNF-α injection. Scale bars: (**A–F**) 10 μm; (**G–Q**) 50 μm.

was determined at 1 mm from the edge of the optic disc in eight distinct areas of 62,500 μm^2 each (two areas per each retinal quadrant). Morphologically distinguishable glial cells, vascular endothelial cells, and presumably displaced amacrine cells with a smaller and denser soma were excluded from the counting. Data from eight areas from each eye were averaged for one eye and a minimum of five eyes per experimental condition were used. All quantification was performed in a masked manner.

Retrograde Labeling of the RGCs

Retrograde labeling of RGCs was performed in a manner similar to that described previously.[25] Briefly, rats were anesthetized by intramuscular injections of a mixture of ketamine-xylazine. Labeling was performed by aspirating the cerebrum and placing a small piece of gel foam soaked in a 6% solution of a neurotracer dye (Fluorogold; Fluorochrome, Denver, CO) at the superior colliculus (SC) bilaterally, 5

days before the intravitreal injection of TNF-α. Eyes obtained from animals at 2 weeks and 2 months after intravitreal injection, were fixed and processed for flatmount preparations. Tracer-labeled RGC counting was performed at 1 mm from the edge of the optic disc under a fluorescence microscope (Carl Zeiss Meditec) using a UV filter in 12 distinct areas of 273,700 μm^2 each (three areas per each retinal quadrant). Distinguishable glial cells (bright and small cells) were excluded from the counting. Data from 12 areas from each eye were averaged for one eye, and a minimum of five eyes per experimental condition were used.

Inhibition of NF-κB

The role of NF-κB was examined using 1 nanomole NF-κB p65 antisense oligodeoxynucleotide (AS ODN), 1 nanomole NF-κB p65 sense ODN, or 0.1 nanomole helenalin (Biomol Research Laboratories Inc., Plymouth Meeting, PA). NF-κB p65 AS ODN and NF-κB p65 sense ODN were synthesized as phosphorothioated ODNs (Molecula, Herndon, VA) and dissolved in 0.1 M PBS. The AS ODN and sense ODN sequences for NF-κB p65 were 5'-AAACAGATCGTCCATGGC-3' and 5'-GCCATGGACGATCTGTTT-3' (GenBank accession no. AF079314; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), respectively.[26] For studies using helenalin, TNF-α and helenalin were dissolved in 1% dimethylsulfoxide (DMSO; Sigma-Aldrich) and diluted to their final concentrations with PBS. The same amount of DMSO in PBS was used as the control.

Statistical Analysis

Data are presented as the mean ± SEM. Differences among groups were analyzed by one-way ANOVA, followed by the Scheffé or Mann-Whitney test. $P < 0.05$ was considered statistically significant.

Results

Morphology of Optic Nerve and Retina after TNF-α Injection

Intravitreal injection of TNF-α induced marked axonal loss and extensive degeneration of the axons, starting at 2 weeks after injection (Figs. 1D–F). After 1 week, splitting of myelin and other degenerative changes involving large-caliber axons were observed (Fig. 1C). After 2 weeks, more extensive changes with prominent axonal degeneration associated with macrophages and a moderate amount of vacuolization were noted (Fig. 1D). Pale and large diameter axons with thin myelin sheaths and darkened degenerated profiles were noted, often near lipid debris-filled macrophages. An increase in vacuolization and axonal degeneration was noted at 1 and 2 months (Figs. 1E, 1F). In contrast to the extensive degenerative changes of the axons in the optic nerve, all retinal layers were relatively well preserved 2 weeks after injection (Figs. 1G–J). At 1 and 2 (not shown) months, a few cells in the RGCL appeared condensed with pyknotic nuclei, whereas the remaining parts of the retina were unremarkable. Immunohistochemistry of MBP showed that there was extensive MBP immunostaining at the postlaminar portion of the optic nerve in normal (Fig. 1K) or PBS-treated eyes (not shown). However, substantial and progressive loss of MBP immunoreactivity was noted in the optic nerves at 1 and 2 weeks after TNF-α injection (Figs. 1L, 1M). TUNEL assay on retinal sections revealed that there were very few positive cells in the nerve fiber layer (NFL) and inner plexiform layer (IPL) at 1 and 2 weeks after TNF-α injection with controls showing no staining (Figs. 1N–Q).

Quantitative analysis of axonal loss showed that the number of axons at 1 week after PBS and TNF-α injection were $220,621 \pm 23,348/mm^2$ and $179,570 \pm 16,732/mm^2$, respec-
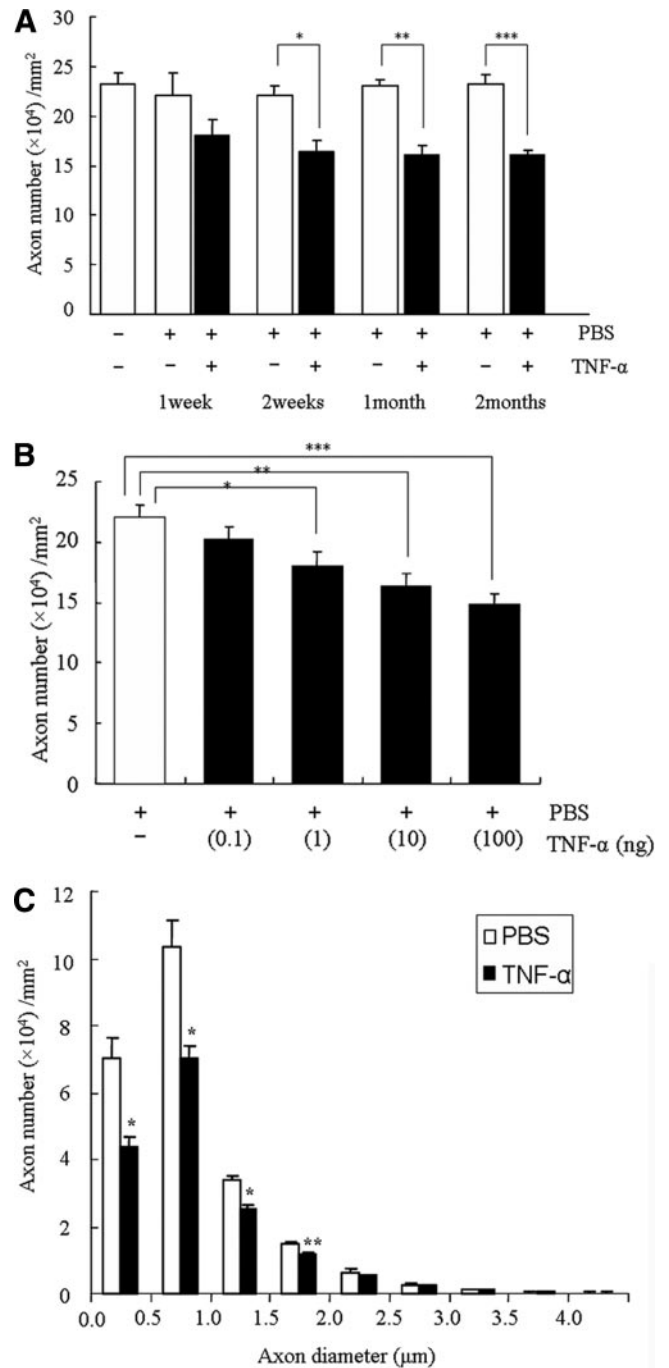

FIGURE 2. Morphometry of optic nerves. All data are expressed as the mean ± SEM. (**A**) Number of axons in the optic nerve after 10 ng TNF-α injection. Significant axonal losses occurred at 2 weeks to 2 months after TNF-α injection ($n = 4$–6; *$P < 0.01$; **$P < 0.05$; ***$P < 0.005$). (**B**) The dose–response relationship of the effect of TNF-α on the number of axons. Significant axonal losses were seen at 1 to 100 ng of TNF-α. Each ($n = 5$–6; *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$). (**C**) Distribution of axon diameters at 2 months after injection by computer-assisted image analysis morphometry ($n = 6$; *$P < 0.005$ vs. PBS-treated eyes; **$P < 0.05$ vs. PBS-treated eyes).

tively (Mann-Whitney test; $P = 0.1416$). After 2 weeks, there was an approximate 30% decrease in the number of axons ($n = 5$; $P < 0.01$ versus PBS-treated eyes). This difference did not significantly change in animals treated for longer periods of
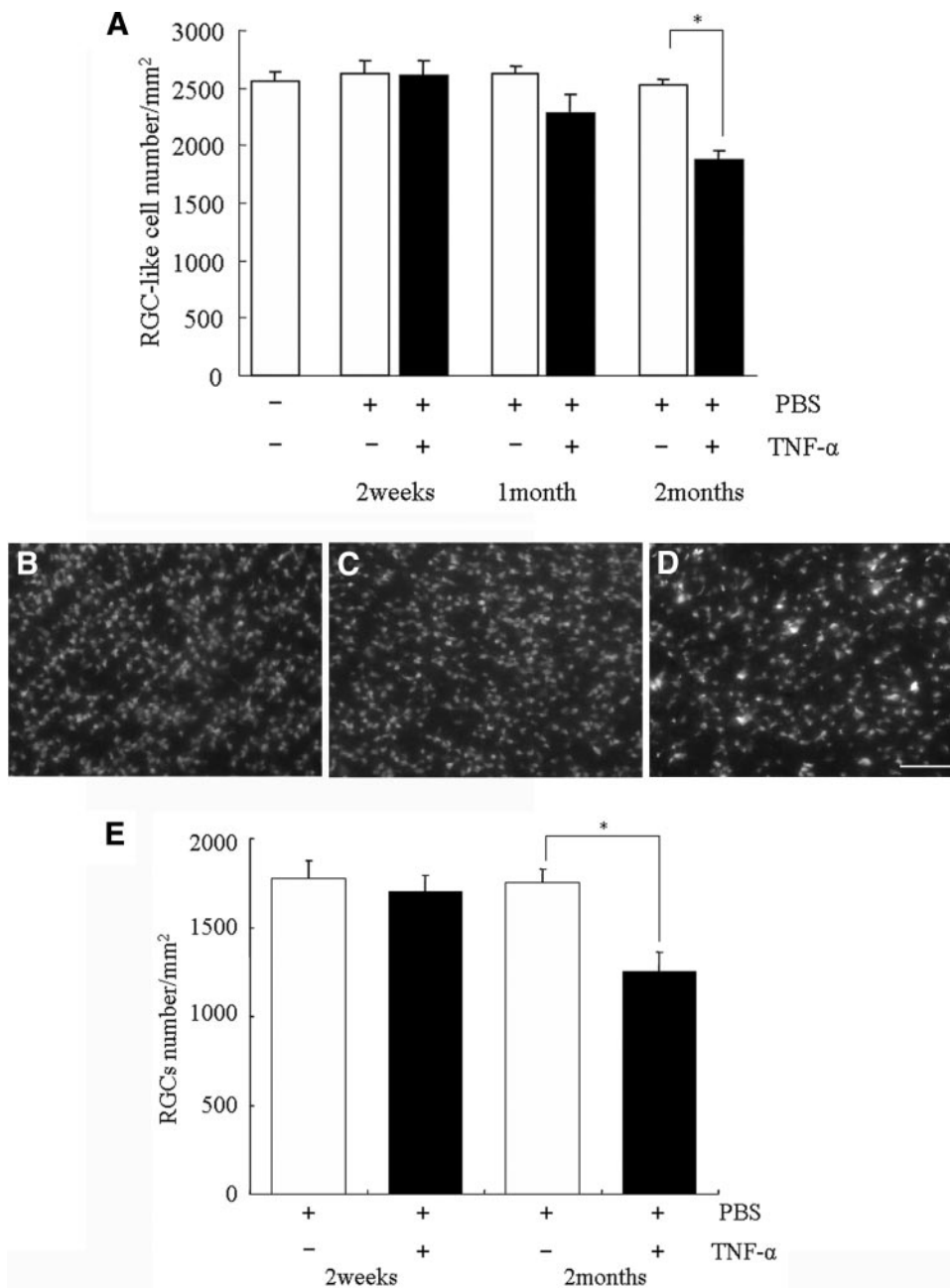
FIGURE 3. Morphometry of cells in the RGCL in wholemount flat preparations. (A, E) Data are expressed as the mean $\pm$ SEM. (A) The number of RGC-like cells estimated by cresyl violet staining after 10 ng TNF-α injection. Significant cell loss occurred at 2 months after TNF-α injection but not at 1 week, 2 weeks, or 1 month. Each column represents the mean $\pm$ SEM ($n = 6$; *$P < 0.005$). (B–D) Neurotracer-labeled RGCs in retinas (B) 2 weeks after PBS injection and (C) 2 weeks and (D) 2 months after 10 ng TNF-α injection. Scale bar, 100 μm. (E) Number of RGCs estimated by neurotracer labeling. There is no significant difference in the number of RGCs between PBS and TNF-α-treated eyes 2 weeks after injection, whereas a significant loss of RGCs was observed 2 months after injection ($n = 5$–6; *$P < 0.05$).

time (i.e., 1 and 2 months; $n = 4$; $P < 0.05$ vs. PBS-treated eyes and $n = 6$; $P < 0.005$ vs. PBS-treated eyes for 1 and 2 months after injection, respectively; Fig. 2A). Moreover, intravitreal injection of TNF-α-induced axonal loss appeared to be dose dependent (Fig. 2B). Of note, the decrease in the number of axons was more prominent, with axon diameters less than 2 μm (Fig. 2C). There was no significant difference in the number of axons between PBS and TNF-α-treated eyes, with axon diameters larger than 2 μm.

Morphometric analysis of cells in the RGCL using wholemounted flat preparations of retinas showed a delayed, time-dependent, cell body loss after the injection of TNF-α when compared with axonal loss and degeneration. In normal retina, the mean RGC-like cell density was 2559 ± 83 cells/mm^2 ($n = 6$; Fig. 3A), as previously reported.[27] Although TNF-α tended to induce a loss of cells at 1 month after injection (RGC-like cell density of PBS and TNF-α-treated eyes were 2617 ± 64 and

2285 ± 167 cells/mm^2, respectively, $n = 6$; Mann-Whitney test; $P = 0.1172$), significant loss of RGC-like cells ($\sim25\%$ loss) was seen only at 2 months after the injection ($P < 0.005$ vs. PBS-treated eyes; Fig. 3A). Moreover, neurotracer labeling (Fluorogold; Fluorochrome) showed no significant difference in the number of RGCs between PBS and TNF-α-treated eyes 2 weeks after injection ($n = 5$–6; Figs. 3B, 3C, 3E), the time at which significant axonal losses were observed in TNF-α-treated eyes. A significant decrease in the number of RGCs was observed at 2 months after TNF-α injection ($P < 0.05$ vs. PBS-treated eyes; Figs. 3D, 3E). Morphometry of immunopositive cells was performed in transverse retinal sections, by using neurofilament[28] and Thy-1 antibodies, which have much higher specificity for RGCs than do displaced amacrine cells. Immunoreactivity of neurofilament was mostly observed in the NFL and RGCL (Fig. 4A) and rarely observed in the inner nuclear layer (INL). In PBS-treated retina, approximately 57.8%
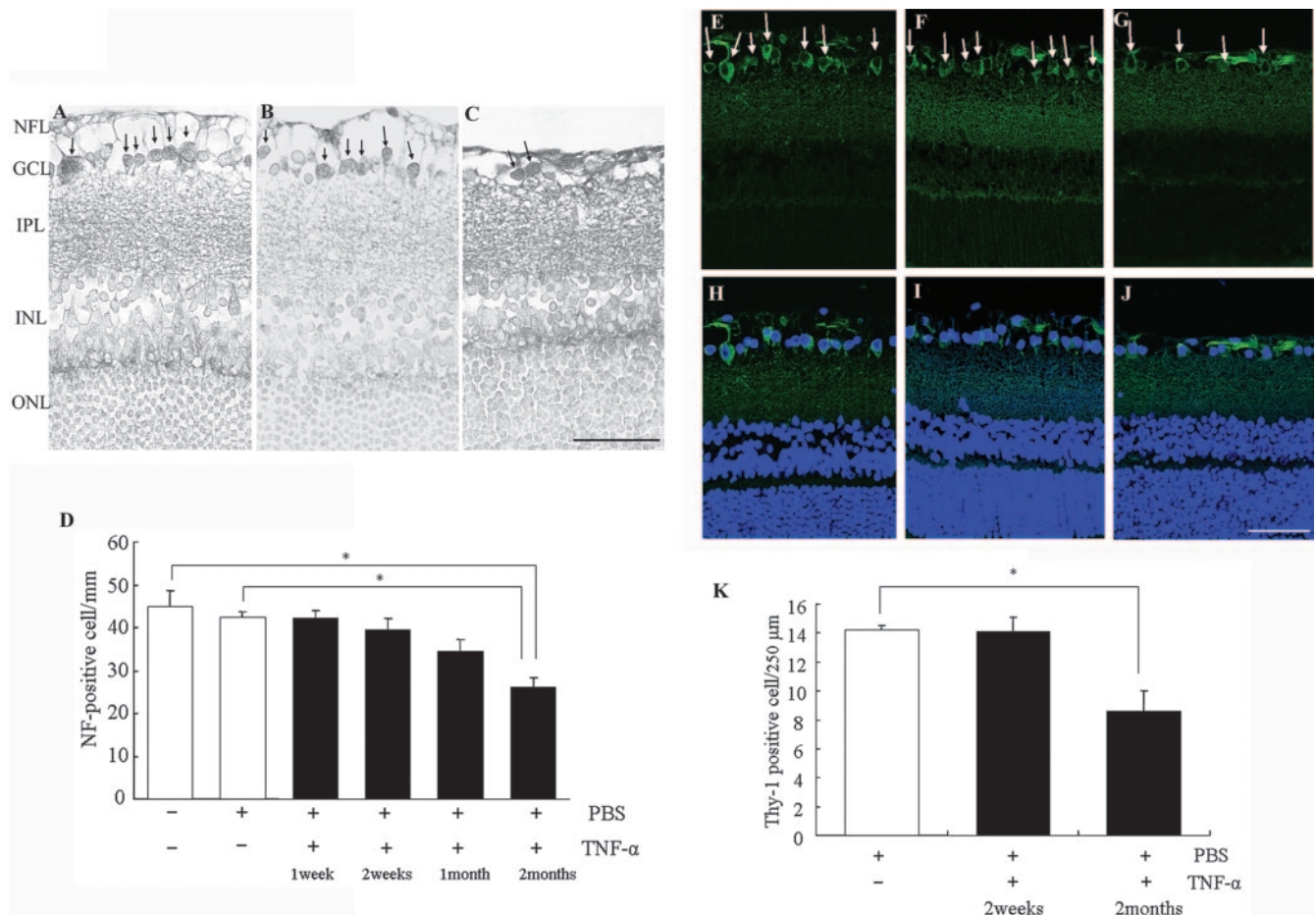
FIGURE 4. Morphometry of cells in the RGCL in retinal transverse sections. (A–C) Neurofilament immunostaining (A) 2 weeks after PBS injection and (B) 2 weeks and (C) 2 months after TNF-α injection. Substantial immunoreactivity was observed in the NFL. *Arrows:* immunopositive cells in the RGCL. (D) The number of neurofilament (NF)-positive cells after 10 ng TNF-α injection ($n = 4$-7; *$P < 0.05$). (E-J) Thy-1 immunostaining with DAPI (H–J), (E, H) 2 weeks after PBS injection, and (F, I) 2 weeks and (G, J) 2 months after TNF-α injection. *Arrows:* Thy-1-positive cells among DAPI-positive cells in the RGCL. (K) Number of Thy-1 positive cells after 10 ng TNF-α injection ($n = 4$; *$P < 0.05$). (D, K) Data are expressed as the mean ± SEM. Scale bars, 50 μm.

of cells in the RGCL were neurofilament positive. (The average number of neurofilament-positive cells and all countable cells in the RGCL were 41.96 ± 3.26/mm and 72.55 ± 3.86/mm, respectively; $n = 5$). Similarly to PBS-treated retinas, there were substantial neurofilament-immunopositive cells 2 weeks after TNF-α injection (Fig. 4B). However, after 2 months, there was a significant decrease in the number of these immunopositive cells in the RGCL (Fig. 4C) but no notable changes in the pattern or intensity of the immunostaining. Quantitative analysis of neurofilament-positive cells showed a similar trend to RGC-like cell counts obtained from wholemounted retinas, in that TNF-α induced a time-dependent decrease in neurofilament-positive cells (Fig. 4D). After 1 month, TNF-α-treated eyes tended to show a marginal loss of immunopositive cells in RGCL (Mann-Whitney test; $P = 0.05$ vs. normal and $P = 0.0833$ vs. PBS-treated eyes). Significant immunopositive cell loss was observed 2 months after TNF-α injection (Mann-Whitney test; $P < 0.05$ vs. normal or PBS-treated eyes). Similar results were obtained with Thy-1-immunopositive cell counting in samples 2 weeks and 2 months after TNF-α injection. There was no significant change in the number of Thy-1-positive cells 2 weeks after TNF-α injection compared with PBS injection (Figs. 4E, 4F, 4H, 4I, 4K). Significant losses of Thy-1 positive cells were seen at 2 months after the injection ($n = 4$; $P < 0.05$; Figs. 4G, 4J, 4K).

Activation of NF-κB p65 and Microglial Cells in Response to TNF-α

NF-κB p65 protein levels increased in optic nerves 1 day after intravitreal TNF-α injection (Fig. 5A). In contrast, there was no notable change of NF-κB p65 levels in retinal samples (Fig. 5C). Densitometry of the NF-κB p65 bands confirmed these observations and demonstrated a significant increase in NF-κB p65 protein levels ($n = 6$–9; $P < 0.05$) in the optic nerve (Fig. 5B) but not in the retina (Fig. 5D).

Immunohistochemistry of NF-κB p65 confirmed an increase of NF-κB p65 immunoreactivity in the optic nerve 1 day after TNF-α injection (Figs. 6B, 6C, 6E, 6F, 6H, 6J, 6L, 6N). Most of the NF-κB p65 immunoreactivity was noted in the distal area of the postlaminar portion of the optic nerve. Double-labeling immunohistochemical studies showed substantial colocalization of NF-κB p65 and ED1 in the optic nerve (Figs. 6A–F) after TNF-α injection. Moreover, most of the NF-κB p65-positive cells showed colocalization with Iba1 after TNF-α injection (Figs. 6G–J). Both ED1 and Iba1 have been used as markers of activated microglia.[29–32] That we also observed substantial Iba1 immunostaining in PBS-treated eyes is consistent with the concept that Iba1 antibody can also recognize resting microglia in the brain.[33] Comparatively, a few cells showed colocalization of NF-κB
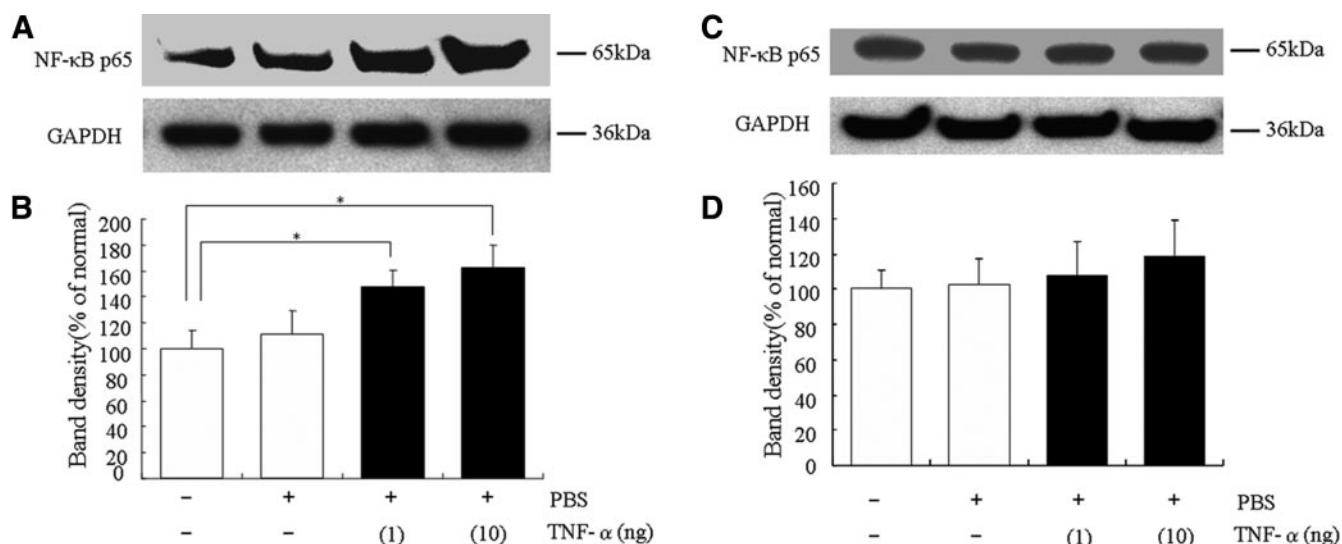
FIGURE 5. Western blot analysis of NF-κB p65 protein levels in rats at 1 day after intravitreal injection. (**A**, **B**) Optic nerves and (**C**, **D**) retinas. Bands at 65 kDa corresponding to the molecular weight of NF-κB p65 were detected in samples from the (**A**) optic nerve and (**C**) retina. GAPDH was used as a loading control. A detectable increase was observed in the optic nerve but not in the retina. Densitometry of the immunoreactive bands in (**B**) the optic nerve and (**D**) the retina. Data are expressed as a percentage of normal. Each column represents the mean ± SEM ($n = 6$-9; *$P <$ 0.05).

p65 and vimentin, a marker of oligodendrocytes and astrocytes, in the optic nerve (Figs. 6K–N).

NF-κB p65 as a Neurodegenerative Modulator in TNF-α-Induced Axonal Degeneration

To examine the role of NF-κB p65 in TNF-α-induced axonal changes, NF-κB p65 AS ODN or helenalin was administered intravitreally, together with TNF-α to block its synthesis or activation, and the number of axons in the optic nerve after 2 weeks was quantified. AS ODN-treated animals showed amelioration of this loss and better preserved nerve fibers (Fig. 7C). Quantitative analysis confirmed the ameliorative effect of AS ODN targeting NF-κB p65 (75.9% protection). The number of axons after PBS, 10 ng TNF-α, or 10 ng TNF-α+AS ODN injection were 220,686 ± 10,163/mm², 163,187 ± 11,211/mm² or 205,847 ± 8,665/mm², respectively ($P <$ 0.05; TNF-α versus TNF-α+AS ODN) (Fig. 7E). In contrast, NF-κB p65 sense ODN did not significantly alter TNF-α-induced optic nerve degeneration as evidenced by either histologic examination (compare Fig. 7D to 7B) or morphometric analysis (Fig. 7E). Helenalin, which selectively alkylates the p65 subunit of NF-κB thereby inhibiting its DNA binding activity,[34] also ameliorated TNF-α-induced optic nerve degeneration, as evaluated by morphometry (Fig. 7F).

Discussion

This present study demonstrates that intravitreal TNF-α injection induced an axonal degeneration with delayed loss of RGC bodies. Concomitant with these axonal changes, an increase in NF-κB p65 protein levels was noted in the optic nerve but not the retina. Inhibition of NF-κB p65 either by AS ODN targeting NF-κB p65 or helenalin, an inhibitor of NF-κB p65 activation, ameliorated the loss of axons in the optic nerve after TNF-α injection. These findings suggest that intravitreal injection of TNF-α induces a primary axonal degenerative event in the optic nerve and that NF-κB p65 may serve as a neurodegenerative modulator.

We postulate that TNF-α induces optic nerve degeneration with subsequent retrograde retinal cell body involvement, as evidenced by qualitative and quantitative changes in the axons of the optic nerve compared with retinal sections. Loss of MBP immunoreactivity, changes in axonal morphology, and decreased axon numbers were observed in sections as early as 2 weeks after treatment with TNF-α. The degree of axonal degeneration reached a plateau and remained constant after 2 weeks of TNF-α treatment in contrast with the retinal cell body changes. Retinal morphology, TUNEL assay, morphometry of neurofilament- or Thy-1-positive cells in retinal sections, and morphometry of cells in the RGCL with flat preparations stained with cresyl violet or neurotracer retrograde labeling displayed unremarkable changes in 2 weeks, but statistically significant changes with increasing length of time after TNF-α exposure. In addition, we were unable to detect any retinal inflammatory reaction. This observation is consistent with previous studies that showed that higher doses of TNF-α (over 50 ng) were necessary for induction of uveitis[35] and that no significant inflammation was noted with doses below 50 ng per eye. These findings are consistent with a previous report suggesting that TNF-α can cause optic nerve axonal degeneration with little involvement of neural retinal damage in rabbits.[20] The decrease in MBP immunostaining indicates demyelination as part of the degenerative process and that not only neuronal damage, but also oligodendrocyte damage may have occurred. These findings are supported by a previous study of the mouse optic nerve showing demyelination at the electron microscopic level.[19] However, to our knowledge, this is the first study to document the temporal changes in the retina as well as the optic nerve after TNF-α exposure. Compared with the optic nerve transsection model (RGC death starts at day 4 and reaches a maximum on day 7 after axotomy), this model displayed milder axonal losses and a very slow RGC loss by morphometry, although both models caused retrograde cell death. Because some ocular degenerative diseases, such as glaucoma, have very slow degenerative processes over an extended period, un-
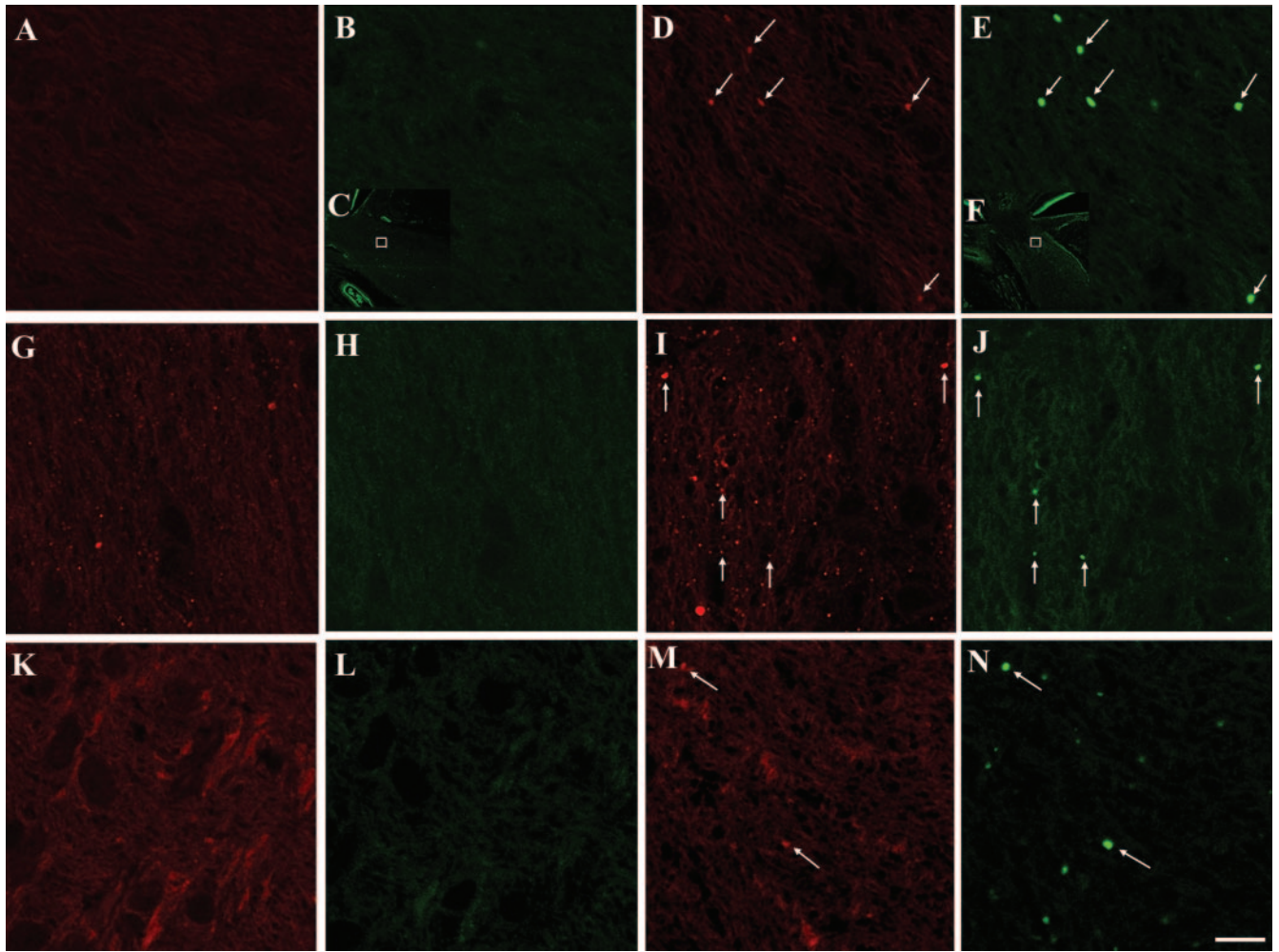
FIGURE 6. **(A–F)** Immunocolocalization of **(B, C, E, F)** NF-κB p65 and **(A, D)** ED1 in the optic nerve. **(A–C)** PBS-treated eye and **(D–F)** 1 day after 10 ng TNF-α injection. Higher magnifications in boxed areas in **(C)** and **(F)** are shown in **(A, B)** and **(D, E)**, respectively. Note extensive colocalization of NF-κB p65 and ED1 in cells scattered in the optic nerve (*arrows*) after TNF-α injection. **(G–J)** Immunocolocalization of **(H, J)** NF-κB p65 and **(G, I)** Iba1 in the optic nerve 1 day after **(G, H)** PBS or **(I, J)** 10 ng TNF-α injection. Substantial Iba1-positive cells are observed in **(G)** PBS or **(I)** TNF-α treated eyes. Note extensive colocalization of NF-κB p65 and Iba1 in cells scattered in the optic nerve (*arrows*) after TNF-α injection. **(K–N)** Immunocolocalization of **(L, N)** NF-κB p65 and **(K, M)** vimentin in the optic nerve 1 day after **(K, L)** PBS or **(M, N)** 10 ng TNF-α injection. A few colocalizations were observed (*arrows*). Scale bars: **(A, B, D, E, G–N)** 10 μm; **(C, F)** 250 μm.

derstanding the mechanisms of both axonal and cell body degeneration in this model may contribute to development of new treatments for these ocular disorders and other neurodegenerative diseases with known axonal involvement.

Previous studies have demonstrated that TNF-α activates NF-κB through TNF-R1, which leads to apoptosis both in neuronal[36] and nonneuronal cells.[11] In contrast, TNF-R2, which has been suggested to exert a cytoprotective effect, also recruits TRAF2 and induces NF-κB activation.[15] Moreover, activation of NF-κB induced by TNF-α is found to be cytoprotective in the peripheral immune system.[37] Thus, it remains unclear why some cells undergo apoptosis and others do not when stimulated by TNF-α. One hypothesis posits that TNF-α may activate both anti- and proapoptotic signaling cascades simultaneously, with the ultimate effect being dependent on the receptor subtype predominantly expressed in individual tissues.[38] In the present study, we observed that NF-κB p65 AS ODN ameliorated TNF-α-induced axonal damages, whereas NF-κB p65 sense ODN did not. Helenalin, an inhibitor of the NF-κB p65 DNA binding

activity,[34] was also effective in ameliorating the degenerative changes. These results strongly suggest a neurodegenerative role of NF-κB p65 in this system and are consistent with other published reports implicating the activation of NF-κB as the cause of neuronal cell death.[39–43] Our results are also consistent with previous reports concerning NF-κB p65 AS ODN in glutamate-induced death in cultured rat cerebellar granule cells,[44] *N*-methyl-D-aspartate (NMDA)-induced retinal neuronal cell death,[26] and acute inhibition of NF-κB by helenalin, delaying neuronal death by inhibition of p53 induction.[45] We cannot exclude the possibility that the failure to detect NF-κB p65 elevation in the retina may be due to the low proportion of RGCs in the retinal cellular population. However, we were unable to detect elevated NF-κB levels in the RGCs or axons by immunohistochemistry (data not shown), in contrast to earlier studies suggesting NF-κB activation in the involved neurons. Alternatively, we observed that most of the NF-κB p65 immunostaining appeared to be present in ED1- and Iba1-positive cells, markers of activated microglial cells,[29–32] in the optic nerve after
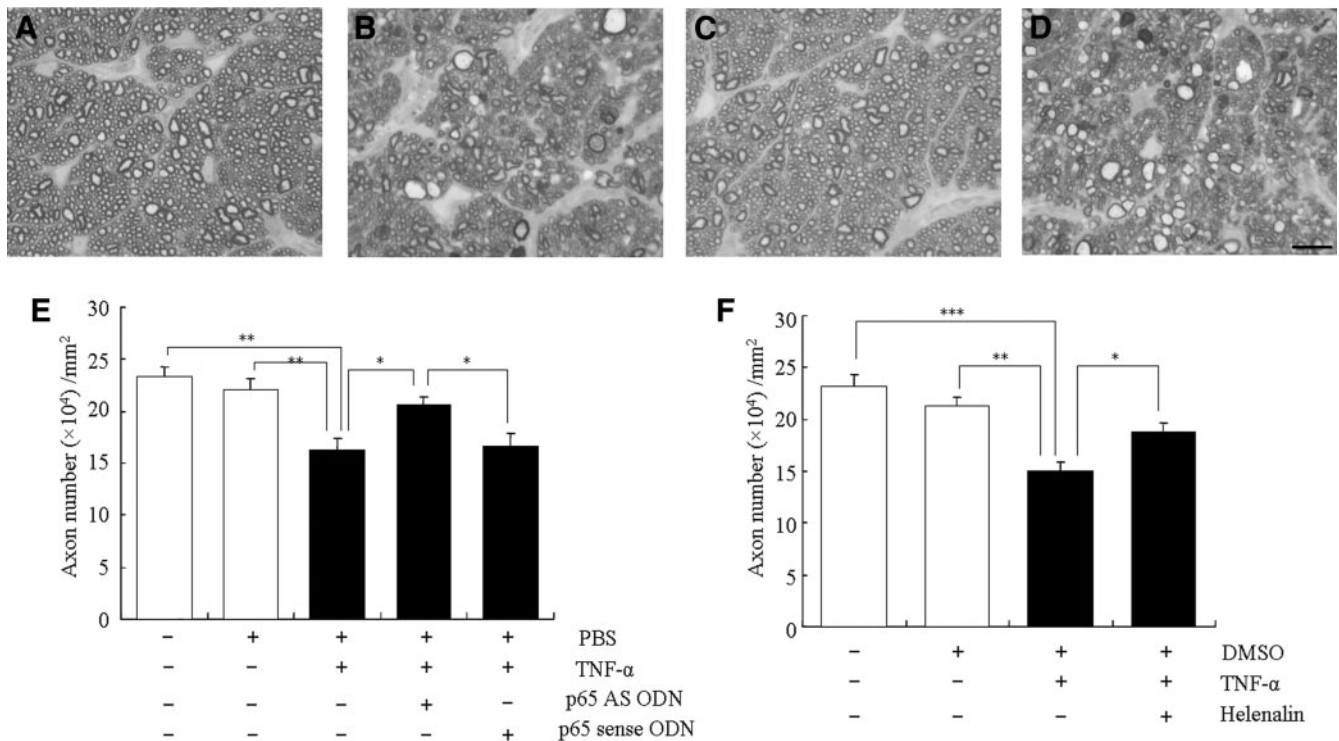
FIGURE 7.   Effects of NF-κB p65 AS ODN and helenalin. Effects of NF-κB p65 AS ODN (1 nanomole) and an NF-κB inhibitor (helenalin; 0.1 nanomole) were evaluated at 2 weeks after 10 ng TNF-α injection. (A–D) Optic nerve cross-sections. (A) PBS-treated eyes; (B) 10 ng TNF-α; (C) 10 ng TNF-α+1 nanomole NF-κB p65 AS ODN; (D) 10 ng TNF-α+1 nanomole NF-κB p65 sense ODN. Scale bar, 10 μm. (E) Effect of NF-κB p65 AS ODN on axon numbers of the optic nerve. Each column represents the mean ± SEM ($n = 4$–6; *$P < 0.05$; **$P < 0.01$). (F) Effects of helenalin on the number of axons of the optic nerve. Each column represents the mean ± SEM ($n = 5$-9; *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$).

TNF-α injection, thus potentially implicating these cells in optic nerve degeneration.

In summary, our findings suggest that TNF-α induces optic nerve degeneration with possible delayed retinal neuronal cell death. There is substantial enhanced expression of NF-κB p65 in the optic nerve, and this increase may be associated with TNF-α-induced axonal degeneration. Moreover, microglial/phagocytic cells may be involved in this process. This model may prove useful for understanding the pathophysiology of axonal neurodegenerative diseases.

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