# Retinal-Cell–Conditioned Medium Prevents TNF-$\alpha$-Induced Apoptosis of Purified Ganglion Cells

*Céline Fuchs, Valérie Forster, Elise Balse, José-Alain Sahel, Serge Picaud, and Luc-Henri Tessier*

**Purpose.** Retinal ischemic processes occurring in glaucoma or diabetic retinopathy induce the secretion of tumor necrosis factor (TNF)-$\alpha$. This cytokine was reported to be either toxic to or protective of retinal ganglion cells (RGCs). In the present study, its effect on RGCs was analyzed in different culture conditions.

**Methods.** Adult rat RGCs were prepared in mixed retinal cell cultures and in purified cultures. They were incubated in normoxic or ischemic conditions, in the presence or absence of TNF$\alpha$ and/or conditioned media isolated from rat retinal glial cell cultures and from adult mixed retinal cell cultures.

**Results.** In mixed retinal cell culture, RGCs were insensitive to TNF-$\alpha$, whereas it induced their degeneration in purified adult RGC cultures. This TNF$\alpha$-elicited toxicity was suppressed by TNF$\alpha$-R1-neutralizing antibodies or caspase 8/10 inhibitors. Analyses of mRNA and protein content in purified RGCs revealed a time-dependent reduction in the expression of the inhibitor of caspase-8, c-FLIP. c-FLIP mRNA was also undetectable after 5 days of culture in the presence of TNF$\alpha$. The retinal cell-conditioned medium protected the RGCs from TNF$\alpha$-induced death and prevented the decrease in c-FLIP mRNA and protein in purified cultures. This medium promoted NF-$\kappa$B translocation in purified RGCs, whereas an NF-$\kappa$B inhibitor induced RGC death in mixed retinal cells.

**Conclusions.** The results confirm that TNF$\alpha$ can induce RGC death by TNF-R1 activation. They indicate, however, that other retinal cells can release a molecule that promotes NF-$\kappa$B translocation in RGCs, the synthesis of the anti-caspase-8, c-FLIP, and thereby prevents TNF$\alpha$-mediated RGC death. (*Invest Ophthalmol Vis Sci.* 2005;46:2983–2991) DOI:10.1167/iovs.04-1177

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In the central nervous system, ischemic, and excitotoxic injuries increase the production of the cytokine, tumor necrosis factor (TNF)-$\alpha$.[1] This cytokine belongs to a TNF superfamily of 19 different protein ligand members—including FASL, TRAIL, and CD30—that mediate their cellular response through 29 receptors of the TNF receptor (TNFR) superfamily.[2] These receptors mediate cellular proliferation, survival, differentiation, and apoptosis.[3] TNF$\alpha$ seems to have a unique and pivotal role in regulating the choice between pro- and antiapoptotic signaling pathways and in the control of cell proliferation and inflammation.[3,4] TNF$\alpha$ exerts its biological functions by interaction with two membrane receptors: TNF receptor types 1 (TNF-R1 or p55) and 2 (TNF-R2 or p75). Although membrane-bound TNF activates both receptors, soluble TNF predominantly stimulates TNF-R1 and has limited signaling capacities for TNF-R2.[4]

In the eye, TNF$\alpha$ has been detected in induced uveitis,[5,6] hereditary retinal degeneration,[7] glaucoma, proliferative vitreoretinopathy, and diabetic retinopathy.[8,9] However, it remains controversial as to whether TNF$\alpha$ is beneficial or detrimental to retinal neurons. In vitro studies have shown that TNF$\alpha$ exerts an apoptotic effect through its receptor TNF-R1 expressed by retinal ganglion cells (RGCs) and that these cells seem to be the first cells damaged after ischemia.[10] Although this has been confirmed in vivo in glaucoma models,[9,11,12] other work has demonstrated TNF$\alpha$-mediated protection of RGCs through TNF-R1 after axotomy[13] or through TNF-R2 in a model of increased intraocular pressure.[12]

A recent report discussing TNF$\alpha$-induced apoptosis versus survival notes two separate complexes that form after trimerization of TNF-R1 in response to TNF$\alpha$.[14] The initial plasma-membrane–bound complex (complex I) consists of TNF-R1, the adaptor TNFR-associated death domain (TRADD), the receptor-1 interacting protein (RIP1), and the TNF receptor associated factor-2 (TRAF2). This complex rapidly signals activation of nuclear transcription factor-$\kappa$B (NF-$\kappa$B). In a second step, TRADD and RIP1 associate with Fas-associated death domain (FADD) and caspases-8/10, forming a cytosolic complex called complex II. In this model complex II induces apoptosis only when complex I-mediated NF-$\kappa$B activation is insufficient and when complex II no longer harbors the caspase-8 inhibitor FLIP.[14] Nevertheless, there is increasing evidence that tissue type and cell environment are important parameters determining the effect of TNF$\alpha$ action.[4] Evidence of these factors in the eye is provided by recent work showing that retinal pigment epithelium (RPE) is resistant to TNF$\alpha$-induced cell death, even after the activation of transcription factor NF-$\kappa$B is specifically blocked.[15] Thus, the specific role of TNF$\alpha$ in the retina remains ambiguous. In particular, the role of TNF$\alpha$ in RGC is yet to be elucidated.

To understand further the molecular mechanisms of the TNF$\alpha$-elicited activity in RGCs, we used three different types of primary cell cultures: mixed retinal cell cultures, purified Müller glial cell cultures, and purified RGC cultures. Glial-derived factors have been shown to promote synapse development in the central nervous system (CNS)[16] and glia-secreted TNF$\alpha$


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may play a role in synaptic plasticity.[17] In this study, preexisting culture techniques[18] were adapted to use only rat adult retinal cells to eliminate the effects of developmental cytokines.

This work presents the mechanisms underlying TNFα-mediated induction of adult RGC death. TNFα did not induce RGC death in mixed retinal cell cultures, whereas it was clearly proapoptotic in purified RGCs. The data suggest that the difference between these conditions relied on a diffusible molecule released by other retinal cells that activate an antiapoptotic pathway in RGCs.

## Materials and Methods

Animals used in these studies were cared for and handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Culture Techniques

**Primary Cultures of Retinal Cells.** Primary cultures of retinal cells were derived from adult rat retinas and prepared by previously described methods, with modifications.[19] Long–Evans rats (2 months old) were anesthetized and the eyes enucleated. The eyes were rinsed with $CO_2$-independent culture medium (Invitrogen, Carlsbad, CA) and retinas were dissected mechanically under a microscope. Retinas were then chopped into small fragments, washed in Ringer's solution without $Ca^{2+}$, supplemented with 0.1 mM EDTA, and incubated in papain solution (1 U/μL; Worthington Biochemicals, Freehold, NJ) previously activated by L-cysteine (0.2 mM; Sigma-Aldrich) in the same buffer for 25 minutes at 37°C. The tissue was dissociated by repeated gentle trituration and seeded in an enzyme-inhibiting solution (Neurobasal-A [NBA]; Invitrogen) supplemented with B-27 (1:50; Invitrogen) and L-glutamine (2 mM; Invitrogen) and denoted $NBA^+$. The cells were seeded into 24-well tissue culture plates, containing coverslips, previously coated with polylysine (2 μg/$cm^2$ for 1 hour) followed by laminin (1 μg/$cm^2$ overnight: both from Sigma-Aldrich). Microscopic inspection by trypan blue exclusion showed that viability was more than 90% at the time of seeding. Seeding was performed at an initial density of $1.25 \times 10^5$ viable cells/$cm^2$, and cells were incubated at 37°C in a humidified atmosphere of 5% $CO_2$-95% air, unless otherwise stated.

**Primary Cultures of Purified Müller Glial Cells.** Retinal Müller glial cells (RMGs) were isolated from trypsin (0.2%) and DNase (50 U/mL)-digested retina, by using a protocol based on those reported previously for use in rabbit and human cells.[20,21] Briefly, after enzymatic digestion, six triturations with a Pasteur pipette were sufficient to dissociate the retina completely. Cell cultures were finally seeded into six-well tissue culture plates previously coated with polylysine and laminin. Cells plated in each 10 $cm^2$-well corresponded to one retina. Then the cells were incubated for 24 hours at 37°C in a humidified atmosphere of 5% $CO_2$-95% air. Dishes were then vigorously rinsed three times with $NBA^+$ medium and reincubated. We kept these cell cultures, which contained only Müller glial cells, for 1 week and changed the medium every 2 days. To perform culture treatments on purified RGCs, conditioned medium from Müller glial cells (RMG CM) was collected every 2 days and immediately added to the RGC culture.

**Primary Cultures of Purified RGCs.** Primary cells cultures were derived from twenty 2-month-old, Long–Evans rat retinas and purified by sequential immunopanning, as described for young rat retinas.[18] RGCs were resuspended in NBA medium (Invitrogen) supplemented with B27 and glutamine ($NBA^+$), and seeding was performed at an initial density of $10^4$ cells/$cm^2$. After 10 days in culture, the cell density was reduced to 10% (1000 cells/$cm^2$) in the control conditions. This decrease in the number of cells was not considered further in the cell quantification.

RGCs were seeded for 10 days into 24-well tissue culture plates containing coverslips previously coated with polylysine (2 μg/$cm^2$ for 1 hour) and then by laminin (1 μg/$cm^2$ overnight). The medium was changed every 2 days.

### Experimental Procedure

**Induction of Ischemia in Retinal Cell Cultures.** Retinal cell cultures were placed for 48 hours in simulated ischemic conditions, 24 hours after cell seeding. Plates were transferred to a control-atmosphere incubator in which oxygen level was reduced to 2%. In these experiments, to simulate ischemia, cultures were exposed to glutamate (1 mM; Sigma-Aldrich) and glucose was replaced with deoxyglucose (1 mM; Sigma-Aldrich).

After 48 hours of simulated ischemia, media were changed to fresh $NBA^+$ and the plates returned to a normoxic (5% $CO_2$-95% air) atmosphere for a further 2 days. The cultures were then fixed in 4% paraformaldehyde and processed for immunocytochemistry.

**Experimental Treatment on Retinal Cell Cultures.** First, to investigate the effect of TNFα, 20, 100, or 200 ng/mL of rat TNFα factor (R&D Systems, Minneapolis, MN) was added to the cultures for 10 days. In addition, to investigate the effect of TNFα on RGC survival, incubations under ischemic conditions were performed in the presence of specific inhibitors. A neutralizing antibody (MAB510; R&D Systems) was used to inhibit TNFα-activity at 10 μg/mL. A selective inhibitor of TNF-R1 activity, TNF-R1-neutralizing antibody (MAB225; R&D Systems), was used at 10 μg/mL. The inhibitors of caspase-10, Z-AEVD-FMK, (APO-76A-045-R020; Apotech, Geneva, Switzerland) and caspase-8, Z-IETD-FMK (APO-76A-038-R020; Apotech) were both used at 3 mM. When the culture medium was changed after 2 days of incubation, TNFα and/or the tested inhibitors were added again to the new medium.

**Experimental Treatment of Purified RGC Cultures.** To investigate the effect of TNFα, 20, 100, or 200 ng/mL was added for 10 days to cell cultures immediately after RGC seeding. Purified RGCs were incubated either with RMG CM or with retinal-cell–conditioned culture medium (retinal CM) for 10 days. Conditioned media were changed every 3 days, and TNFα was added again to the new medium.

### Immunocytochemistry

Cells from pure Müller glial cell or pure RGC cultures were fixed with 4% paraformaldehyde solution for 15 minutes at room temperature. After they were washed with PBS, they were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature and treated with 0.1% bovine serum albumin, 0.1% Tween 20, and 5% goat serum for 1 hour to block the nonspecific binding sites. Three coverslips from retinal cell culture or pure RMG cell culture were incubated with polyclonal rabbit antibody against TNFα (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) and three coverslips from the retinal cell culture or pure RGC culture were incubated with a polyclonal goat antibody against TNF-R1 (1:100; Santa Cruz Biotechnology) overnight at 4°C. The samples were then washed and incubated with the appropriate secondary antibodies conjugated with Alexa-594 (Molecular Probes, Inc., Eugene, OR).

For localization of TNFα and TNF-R1 expression, the cells were double immunolabeled with specific cell markers. For double immunofluorescence labeling, after the fixation, permeabilization and blocking steps, the cultures were incubated with a mixture of two antibodies (TNFα and vimentin; TNF-R1 and NF200). After they were washed, the cells were incubated with a mixture of corresponding Alexa-488 (green emission)- and Alexa-594 (red emission)-conjugated secondary antibodies (Molecular Probes, Inc.) for 1 hour at 37°C. Negative control experiments were performed by incubating the cells with each primary antibody, followed by the inappropriate secondary antibody to determine that each secondary antibody was specific to the species it was made against. Fluorescence labeling was observed with a microscope (Optiphot 2; Nikon, Tokyo, Japan) under epifluorescence illumination. All images were acquired with a charge-coupled device

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(CDD) color camera (Cool-Snap FX; Photometrics, Tuscon, AZ) and analyzed on computer (Metaview software; Ropper Scientific, Inc.).

To study NF-κB translocation, purified RGCs were incubated in the presence or absence of retinal CM for 10 days. The medium was changed every 3 days. The cultures were fixed with methanol for 2 minutes at −20°C, treated with 0.1% bovine serum albumin, 0.1% Tween 80, and 5% goat serum for 1 hour and incubated with the polyclonal rabbit antibody against NF-κB p65 (1: 200; Santa Cruz Biotechnology) overnight at 4°C and subsequently with the goat anti-rabbit antibody conjugated with Alexa-594 (Molecular Probes, Inc.).

## Assessment of Cellular Loss In Vitro

**Immunocytochemistry.** Survival RGCs were identified through their intense NF immunoreactivity and distinctive morphology (large, rounded cell body; laterally displaced nucleus; and extensive neurites). Cells were fixed, permeabilized, and blocked, as described earlier. Coverslips were then incubated with primary antibody NF200 (rabbit polyclonal antibody, 1:1000; Sigma-Aldrich) overnight at 4°C, washed, and exposed for 1 hour to goat anti-rabbit secondary antibody and 4′,6′-diamino-2-phenylindole (DAPI). Coverslips were then washed thoroughly, mounted, and viewed with a photomicroscope (Optiphot 2; Nikon) equipped with Nomarski differential interference optics and epifluorescence illumination.

**Cell Counting and Statistical Analyses.** RGC survival was assessed by counting the NF200-positive immunolabeled cells after a period of ischemia, during which cells exhibiting continuous plasma membranes with no signs of vacuolation and well-developed neuritic processes were scored as viable at the time of fixation. Cells showing nuclear pyknosis or fragmentation, as visualized by DAPI staining, were considered dead and were excluded from the counts.

All experiments were performed in triplicate, and cell counting was performed on four coverslips for each experiment. Cells on each coverslip were counted under a 20× objective. Data are expressed as the total number of RGCs per coverslip.

Statistical analyses were performed by computer with an analysis of variance (ANOVA) software package followed by the Newman-Keuls multiple-comparison test. $P < 0.05$ was considered statistically significant. For the statistical analysis of conditioned medium, Student's *t*-test was used. These analyses were performed on raw data, and graphic representation of the results were normalized to control values (expressed as 100%) for each experimental series (to minimize variation) and are represented as the percentage ± SEM.

## RNA Extraction and RT-PCR

Total RNA was extracted from approximately $5 \times 10^4$ RGCs by using an RNA purification system (Versagene; Gentra, Minneapolis, MN). Briefly, all culture media were removed from the wells and 400 μL of lysis solution was added. To achieve complete homogenization, the lysate was passed through a 26-gauge needle eight times, and then the purification protocol was performed. Of the 70 μL of elution solution used to recover RNA from the purification column, 15 μL was used for reverse transcription. Three pairs of oligonucleotides were placed in 8 μL of water and added to the 15 μL containing the RNA. The 23 μL was placed at 65°C for 3 minutes and then placed on ice. Then, 25 μL of 2× reaction buffer, and 2 μL of polymerase was added to the sample (SuperScriptIII RT/Platinum *Taq*; Invitrogen). The cDNA synthesis was achieved in a 30-minute incubation at 50°C followed by one cycle of denaturation at 94°C for 2 minutes. A first PCR amplification was achieved with the first set of oligonucleotides (described later) after 37 cycles (94°C for 30 minutes, 52°C for 45 minutes, 68°C for 45 minutes) and a final extension of one cycle at 68°C for 5 minutes. This first PCR solution (diluted 1:100) was reamplified using a different set of oligonucleotides (shown later). This diluted primary PCR solution (5 μL) was used for a second *Taq* PCR (Invitrogen) amplification consisting of one cycle of denaturation at 94°C for 2 minutes, followed by 35 cycles (94°C 30 minutes, 55°C 45 minutes, 72°C 45 minutes) and one cycle at 72°C for 5 minutes. This second amplification was performed with a single set of oligonucleotides. Ten microliters of the 50-μL PCR product was analyzed on ethidium bromide-stained agarose gel. To detect a possible genomic contamination the SuperScriptIII was omitted and replaced by *Taq* Platinum alone.

Accession numbers of the sequences used in this study are NM057138 for rat FLIP mRNA, NM138860 for rat melanopsin mRNA, and X150 for rat acidic ribosomal phosphoprotein PO mRNA. The locations of the oligonucleotides used for RT/PCR are given with respect to these sequences. Sequences of the oligonucleotides were as follows: (1) the caspase 8 inhibitor FLIP (Cflar) mRNA, first RT- PCR (494-bp fragment): reverse, (+692) CACTGGCTCCAGACTCACC (+674); forward, (+199) TAAGTGAGAGAGGCCAGCTC (+218); second PCR (302-bp fragment): reverse, (+591) TGGACTGCGTGTACTTCTGG (+572); forward, (+290) AGATAAAGCAGCCGTGGAGG (+309); (2) melanopsin (opsin 4) mRNA, first RT-PCR (536-bp fragment): reverse, (+1494)TGCTTGCTGGGCAGCTCCCC (+1475); forward, (+959) GCTACATCTTCATCTTCAGGGCC (+981); second PCR (362-bp fragment): reverse, (+1460) TCTGTGTCTGTCCAGCCCAC (+1441); forward, (+1099) CTCTTTGTGCTGTCCTGGGC (+1118); and (3) acidic ribosomal phosphoprotein PO mRNA, first RT-PCR (403-bp fragment): reverse, (+871) TTGACCTTTTCAGCCAGTG (+852); forward, (+469) CGAGAAGACCTCTTTCTTCCAA (+490); second PCR (259-bp fragment): reverse, (+857) CCAGTGGGAAGGTGTAGTCAGT (+836); forward, (+608) AACATCTCCCCCTTCTCCTTC (+628).

## Western Blot Analysis

RGCs were cultured in the presence or absence of retinal CM for 10 days. Cells were washed twice with PBS and prepared in lysis buffer (20 mM $Na_2PO_4$, 250 mM NaCl, 30 mM NaPPi, 0.1% NP40, 5 mM EDTA, and 5 mM dithiothreitol [DTT]) containing a protease inhibitor cocktail (Sigma-Aldrich). Cell debris and nuclei were removed by centrifugation at 10,000*g* for 5 minutes, and the protein concentration was determined by Bradford assay (Sigma-Aldrich). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Immobilon; Millipore, Bedford, MA) by electroblot. Nonspecific binding sites were blocked by incubation in PBS containing 0.1% Tween-20 and 5% dry milk for 2 hours at room temperature. The membranes were then incubated overnight with rabbit polyclonal antibody directed against c-FLIP (1:1500; Biocarta, San Diego, CA) or with mouse monoclonal antibody directed against actin (1:5000; Chemicon, Temecula, CA) at 4°C. Bound primary antibodies were visualized with horseradish-peroxidase–conjugated goat anti-rabbit-IgG or goat anti-mouse-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham, Piscataway, NJ).

# Results

## In Vitro Cellular Localization of TNFα and TNF-R1

Immunolabeling using specific markers in vitro was performed on adult rat retina culture systems, to identify TNFα-secreting cells and those expressing TNF-R1. In retinal cell cultures, TNFα-positive cells were also labeled with the vimentin antibody, indicating that they were glia (Figs. 1A–C). By contrast, TNF-R1 receptors were located in neuronal cells with long processes. Double immunolabeling with the antibody directed against the neurofilament NF200 indicated that these TNF-R1-positive cells were RGCs (Figs. 1D–F). Expression of TNFα in glial cells was confirmed in pure adult Müller glial cell cultures in which all cells showed the TNFα labeling (Figs. 1G–I). Expression of TNF-R1 in RGCs was confirmed in pure adult RGC culture in which all cells showed the TNF-R1 labeling (Figs. 1J–L).

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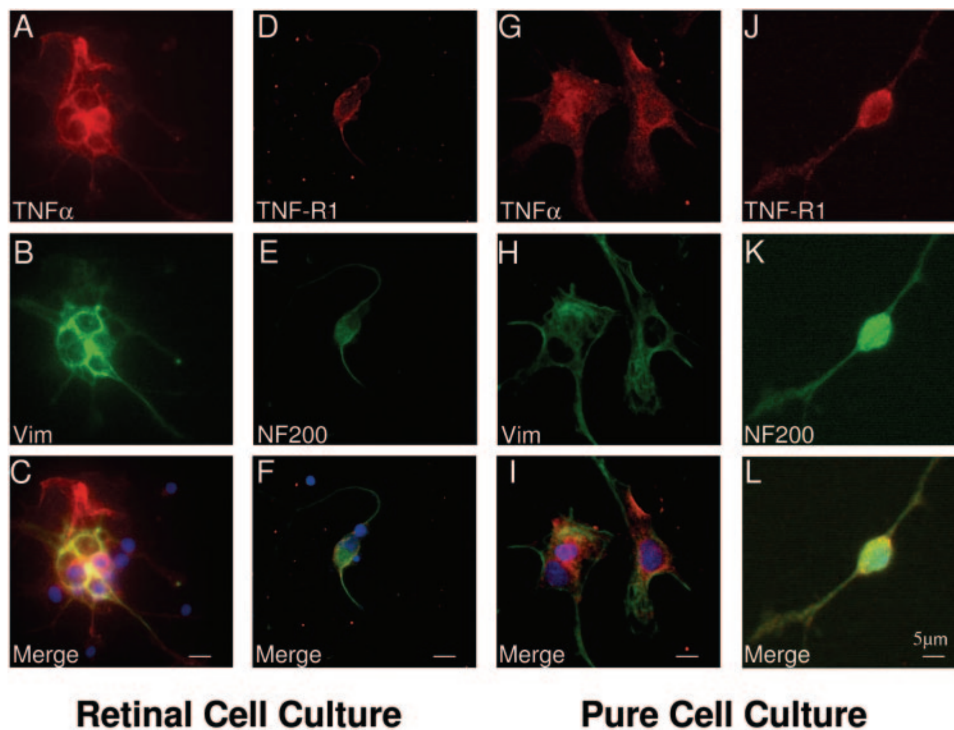


**FIGURE 1.** TNFα and TNF-R1 localization using double immunofluorescence labeling. (**A–C**) TNFα expression in retinal cell cultures: immunostaining for TNFα (**A**), vimentin (**B**), and colocalization of TNFα and vimentin by DAPI staining (**C**). (**D–F**) TNF-R1 expression in retinal cell cultures: immunostaining for TNF-R1 (**D**), NF200 (**E**), and colocalization of TNF-R1 and NF200 by DAPI staining (**F**). (**G–I**) TNFα expression in pure retinal Müller glial cell cultures: immunostaining for TNFα (**G**), vimentin (**H**), and colocalization of TNFα and vimentin by DAPI staining (**I**). (**J–L**) TNF-R1 expression in pure RGC cultures: immunostaining for TNF-R1 (**J**), NF200 (**K**) and colocalization of TNF-R1 and NF200 by DAPI staining (**L**).

## Effect of TNFα on RGCs in Normal and Ischemic Retinal Cell Cultures

TNFα's impact was first evaluated in retinal cell cultures by using different concentrations of TNFα under normoxic control conditions. Cell survival was assessed by counting RGCs immunolabeled by the NF200 antibody. No statistically significant difference was observed (Fig. 2A) in the concentration range used in this study, and TNFα did not induce any RGC death.

The effect of TNFα was then assessed in ischemic conditions. Retinal cells were cultured in an incubator where oxygen tension was less than 2%, and the medium was deprived of glucose but contained 1 mM deoxyglucose plus 1 mM glutamate. After 48 hours, the cells were placed in a medium containing glucose in an incubator with 21% oxygen. Most RGCs (78% ± 1.6%, $n = 3$) did not survive these ischemic conditions. To determine the role of TNFα in this massive RGC death in these conditions of metabolic stress, ischemic cultures were further incubated in the presence of specific inhibitors. Either TNFα (Fig. 2B)- or TNF-R1 (Fig. 2C)-neutralizing antibodies were used. Neutralizing TNFα resulted in a 1.9-fold increase in RGC survival (42% ± 3.2%, $n = 3$) when compared with the ischemic conditions (22% ± 1.6%, $n = 3$). Similarly, neutralizing TNF-R1 produced a 2.2-fold increase in RGC survival (39% ± 4.6%, $n = 3$). These results are consistent with the notion that, during ischemia, TNFα can contribute to adult RGC death after TNF-R1 activation.

## Effect of TNFα on Pure RGCs Cultured in Different Media

Adult RGCs were purified by sequential immunopanning to confirm that TNFα is active only when RGCs are exposed to ischemic conditions. Contrary to expectations, results obtained with these cultures (Fig. 3A) demonstrated that adult RGCs were TNFα-sensitive in normoxic conditions, as 63% ± 1.8% ($n = 3$) of RGCs were lost upon application of TNFα. Neutralizing TNF-R1 antibody produce a 1.7-fold increase in RGC survival (64% ± 2.2%, $n = 3$; Fig. 3A).

RGC resistance to TNFα in retinal cell culture could be explained by the presence of a survival factor in the medium. To test this possibility, addition of TNFα was performed on purified RGCs cultured in $NBA^+$ medium or in different conditioned media obtained from either retinal or Müller glial cell cultures. In the absence of TNFα (Fig. 3B), a strong increase in survival rate was observed with RMG CM (648% ± 108%, $n = 3$) and, to a lesser extent, with retinal CM (303% ± 16%, $n = 3$), when compared with $NBA^+$ control medium. The results shown in Figure 3C indicate that retinal cell culture media almost completely inhibited TNFα-mediated RGC death (96% ± 4.5% rate of survival; $n = 3$), whereas Müller glial cell culture media did not protect RGCs against TNFα (65% ± 8.9% rate of survival; $n = 3$).

## Survival Pathway Activation

**NF-κB Involvement.** Inhibition of cell death specifically induced by TNFα has been reported to occur through NF-κB activation.[22] NF-κB inhibitor was therefore used to determine whether NF-κB was a key regulator of the cell death observed in our system in promoting RGC survival. A phenolic antioxidant such as butylated hydroxyanisol (BHA) can suppress or at least attenuate NF-κB activation.[23] When such a compound was added to retinal cell normoxic cultures (Fig. 4), RGC death was observed in the presence (36% ± 1.7%, $n = 3$) or absence (43% ± 4.3%, $n = 3$) of neutralizing TNFα antibody. The difference between these two conditions is not statistically significant. When TNFα was added to the culture concomitantly with BHA, the cell loss increased further to 73% ± 2.7% ($n = 3$). These results were consistent with a contribution of the transcription factor NF-κB to rat retinal cell survival and especially to the prevention of their TNFα-induced cell death.

To investigate whether NF-κB activation is involved in RGC neuroprotection against TNFα-mediated toxicity by retinal CM, its subcellular localization was examined in purified RGCs in

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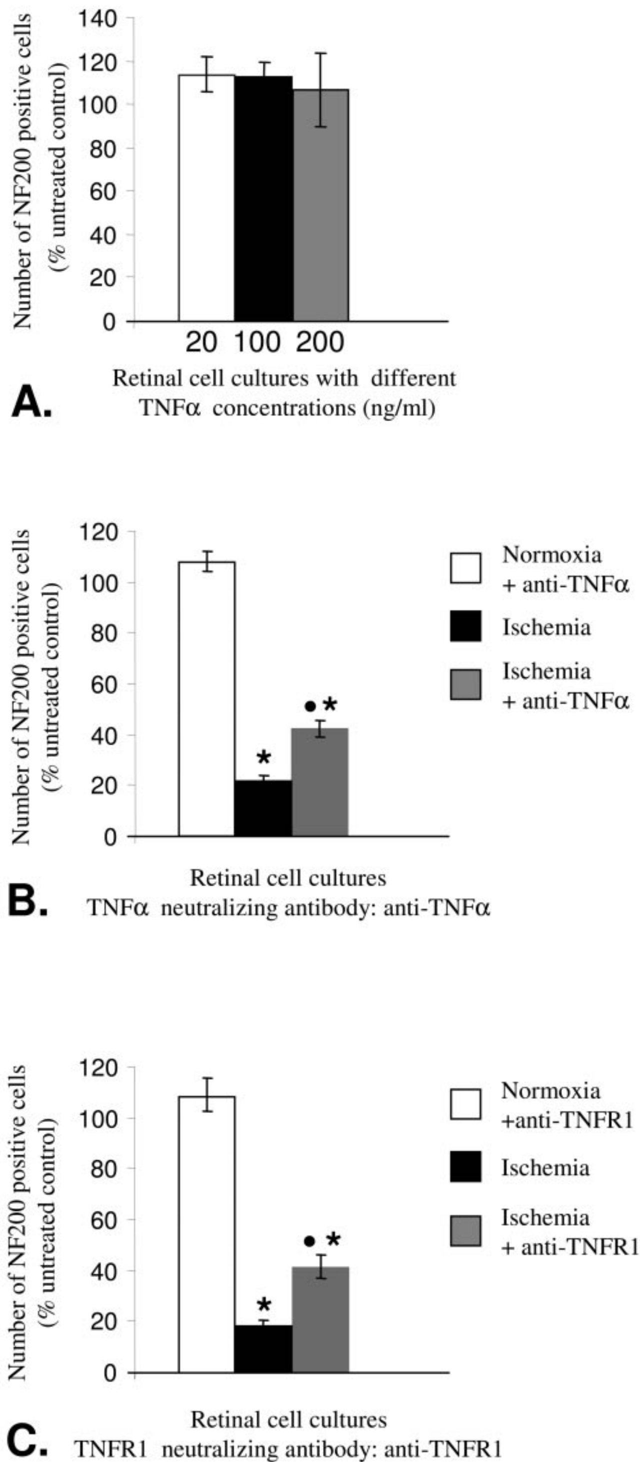


**Figure 2.** Analyses of RGC survival in retinal cell cultures under normoxic or ischemic conditions. (**A**) Effect of three different concentrations of TNFα on normoxic cultures. Effect of (**B**) TNFα- or (**C**) TNF-R1-neutralizing antibody on normoxic or ischemic conditions and cell death obtained in ischemic conditions without neutralizing antibodies. Specific antibodies were used at the same concentration (10 μg/mL) and resulted in increased rates of RGC survival from 22% to 42% (**B**) and from 17% to 39% (**C**). RGC survival was assessed by quantitative analysis of NF200-positive cells. ANOVA and Newman-Keuls multiple-comparisons test (*$P < 0.05$, compared with untreated control cultures; •*$P < 0.05$, compared with ischemic control cultures).

the presence or absence of retinal CM. Figures 4B–G illustrate that the cytoplasmic NF-κB in purified RGCs (Fig. 4B–D) was translocated in the RGC nuclei in the presence of retinal CM (Fig. 4E–G). This NF-κB translocation induced by the retinal CM in purified RGCs is consistent with the implication that NF-κB is neuroprotective against TNFα-mediated toxicity in RGCs.

**c-FLIP Recruitment.** Because the prevention of the TNFα toxicity was reported to involve the NF-κB activation and a subsequent synthesis of the caspase-8 inhibitor,[14] c-FLIP expression was therefore evaluated in RGCs at the mRNA and protein level. In purified RGCs, c-FLIP mRNA were present

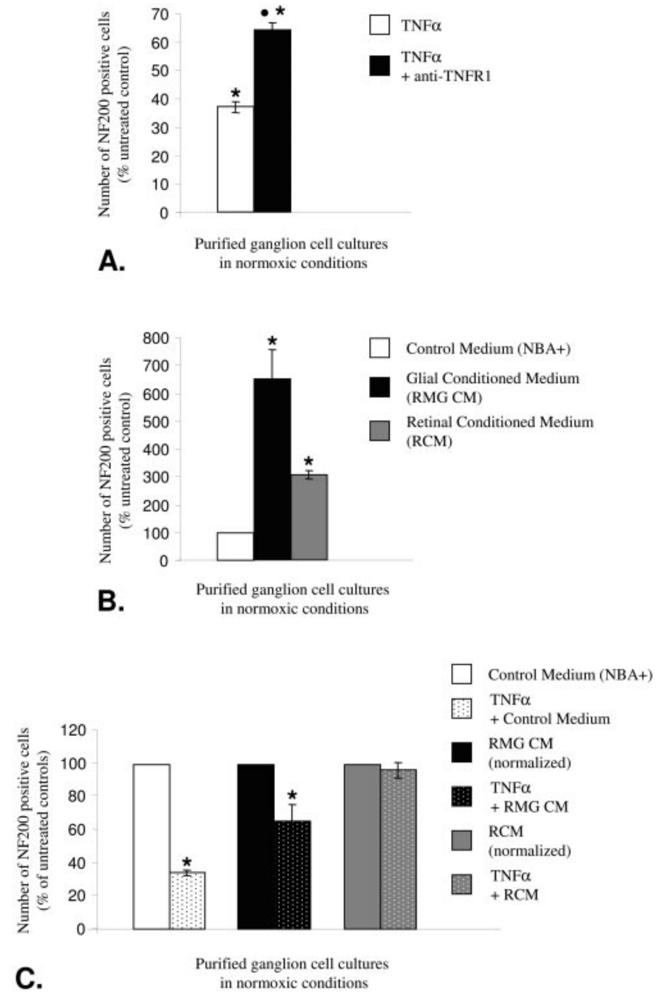


**Figure 3.** Analyses of survival cells in purified RGC cultures under normoxic conditions. (**A**) Effect of TNFα on RGC cultures after 10 days, using either 200 ng/mL TNFα- or 200 ng/mL TNFα+10 μg/mL TNF-R1-neutralizing antibody. RGCs were cultured in NBA$^+$. RGC survival was assessed by quantitative analysis of NF200-positive cells. ANOVA and Newman-Keuls multiple-comparisons test (*$P < 0.05$, compared with untreated control cultures; •*$P < 0.05$, compared with TNFα treatment cultures). (**B**) Percentage of RGC survival rate when cultured either with RMG CM or retinal CM. The increase in survival rate observed with both media were compared with survival in NBA$^+$ control medium. ANOVA and Newman-Keuls multiple-comparisons test (*$P < 0.05$, compared with untreated control cultures). (**C**) Inhibition of TNFα-mediated RGC death observed with NBA$^+$ medium, with RMG CM or with retinal CM. The TNFα concentration was 200 ng/mL. Student's *t*-test for these experiments (*$P < 0.05$). Each condition was compared with its appropriate control: NBA$^+$ medium, RETINAL CM, or RMG CM.

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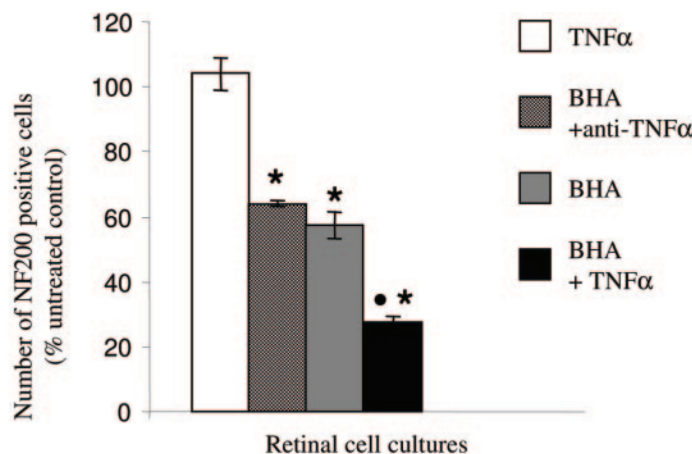


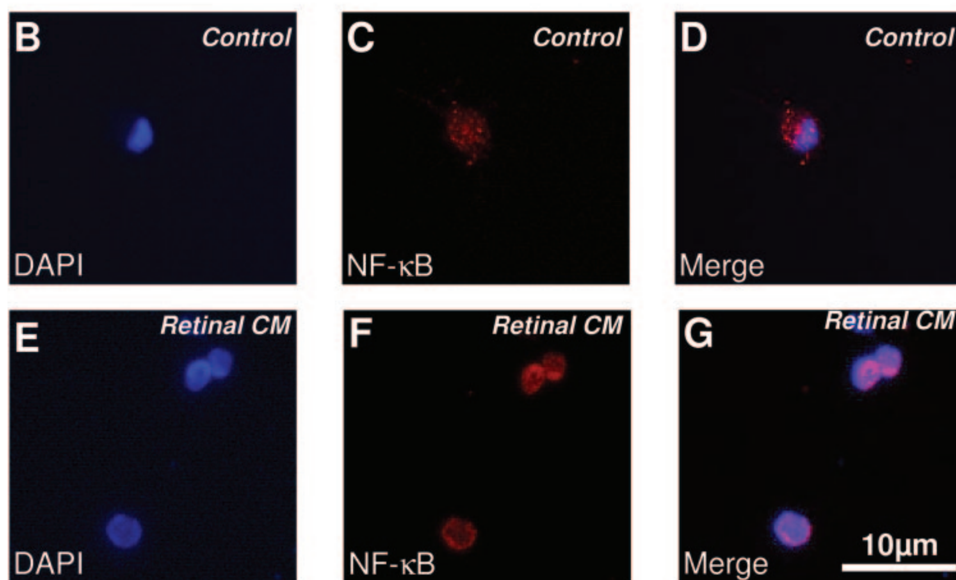


**Figure 4.** Role of NF-κB in RGC neuroprotection. (**A**) Retinal cell cultures were incubated in normoxic conditions for 10 days with 200 ng/mL TNFα; 1 μM BHA, an NF-κB inhibitor; 1 μM BHA+10 μg/mL TNFα-neutralizing antibody; or 1 μM BHA+200 ng/mL TNFα. RGC survival was assessed by quantitative analysis of NF200-positive cells. ANOVA and Newman-Keuls multiple comparison test (*$P < 0.05$, compared with untreated control cultures; •*$P < 0.05$, compared with BHA-treated cultures). (**B–G**) NF-κB p65 subcellular distribution in purified RGCs in the absence (**B–D**; control) or presence of retinal CM (**E–G**) after a 10-day incubation. Cell nuclei were revealed by DAPI nuclear labeling (**B**, **E**). The NF-κB p65 immunolabeling (**C**, **F**) is merged with DAPI labeling (**D**, **G**) to illustrate the NF-κB p65 translocation in retinal CM (**G**).

after 5 days in vitro but disappeared after 10 days, whereas the melanopsin or the ribosomal protein mRNAs were expressed continuously (Fig. 5A). When RGCs were cultured in the presence of retinal CM, which induced NF-κB translocation, c-FLIP expression was recovered at the mRNA level. This recovered c-FLIP expression induced by the retinal CM was confirmed by Western blot analysis at the protein level (Fig. 5B). In the presence of TNFα, both c-FLIP and melanopsin mRNA expression was suppressed after 5 days in culture and recovered when retinal CM was added to the TNFα (Fig. 5C). These observations indicated that the retinal CM maintained c-FLIP expression in purified RGCs.

**Inhibition of Caspase-8 and -10.** Results obtained with the NF-κB inhibitor and studying c-FLIP expression imply that caspase-8 and -10 are probably recruited at the very beginning of the death process (see the Discussion section). This hypothesis was tested by using inhibitors of caspase-10 and -8. In ischemic retinal cell culture, the caspase-10 inhibitor (Z-AEVD-FMK) resulted in a 1.9-fold increase in RGC survival. The increase in RGC survival (Fig. 6A) using the anti-caspase-10 inhibitor (26% ± 3.1%, $n = 3$) was similar to the one previously observed with the use of anti-TNFα (+22%)- or anti-TNF-R1 (+17%)-neutralizing antibodies (Figs. 2B, 2C). Similarly, pure RGCs were rescued from the TNFα toxicity by the anti-caspase-10 inhibitor (Fig. 6B), resulting in a 1.7-fold increase in RGC survival. This protection from apoptosis using anti-caspase-10 (67% ± 5.6%, $n = 3$) is similar to that observed using the TNF-R1 neutralizing antibody (Fig. 3A). When the caspase 8 inhibitor (Z-IETD-FMK) was applied on pure RGCs incubated in the presence of TNFα, it increased RGC survival by a 2.7-fold (86% ± 7.4%, $n = 3$; Fig. 6C). These results confirmed that TNF-mediated toxicity of RGCs involved caspase-8 and -10 activations.

## Discussion

### TNFα Expression and TNF-R1 Activation

In this study, TNFα activities are shown to be dependent not only on the cell types present in the culture, but also on their

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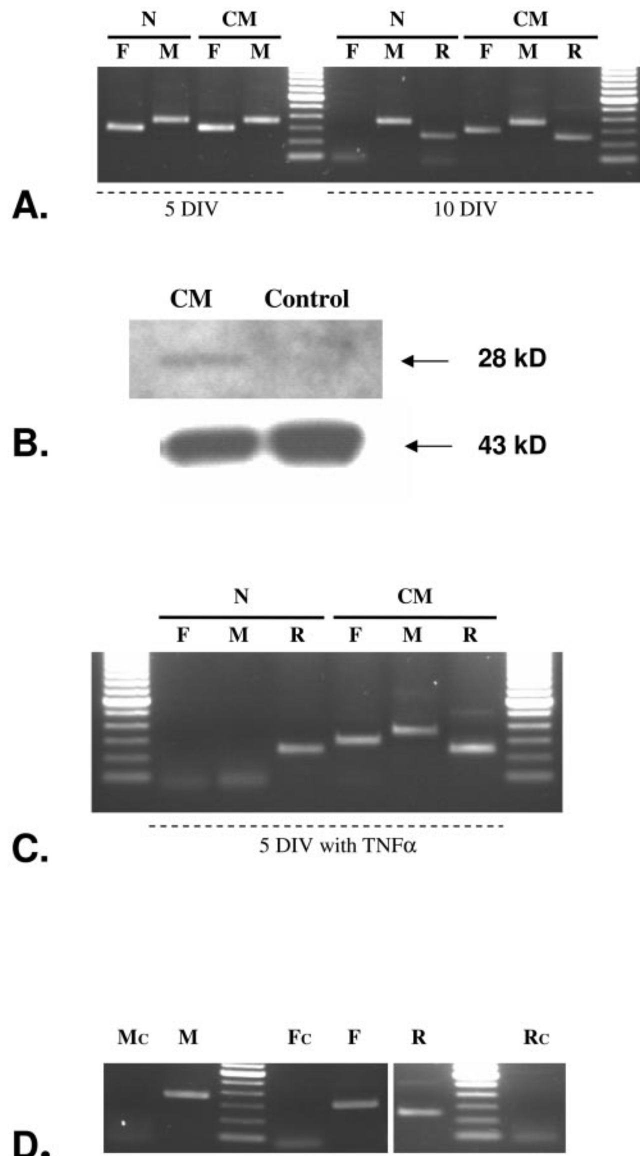


**FIGURE 5.** RT-PCR and Western blot analysis of c-FLIP in purified RGCs. (**A**) Expression of c-FLIP (F), melanopsin (M), and ribosomal protein (R) mRNAs in pure RGCs cultured in either normal $NBA^+$ (N) or retinal CM (CM) for 5 (5 DIV) or 10 (10 DIV) days. Note the disappearance of the c-FLIP-amplified DNA product after 10 DIV. A 100-bp DNA standard marker ladder and DNA products were resolved in a 1.2% agarose gel and visualized with ethidium bromide. (**B**) Western blot probed with c-FLIP and actin antibodies on protein extracts from pure RGCs cultured for 10 days in the absence (control) and presence of retinal CM (CM). The 28-kDa band corresponds to the short c-FLIP form and the 43-kDa band to actin. Note the disappearance of the c-FLIP band in the absence of retinal CM. (**C**) Pure RGCs cultured for 5 days in the presence of 200 ng/mL of TNFα in $NBA^+$ (N) or in retinal CM (CM). (**D**) Internal controls for PCR obtained from RNA extracted from pure RGC cultured in normal NBA+ medium for 5 days. Mc, Fc, and Rc correspond to amplification performed without reverse transcriptase for melanopsin (M), c-FLIP (F), and ribosomal protein (R).

cellular interactions and on their physiological state. Mixed retinal cell cultures from the adult rat retina were first investigated for the presence of TNFα-secreting cells and those containing the TNF-R1 receptor. The glial origin of TNFα with the presence of TNF-R1 on RGCs is in agreement with a previous study performed on human glaucomatous eyes.[9] In in vitro conditions, glial cells have even been reported to synthesize the TNFα and release it under simulated ischemia or elevated hydrostatic pressure.[10] As the TNF-R1 receptor was expressed in cultured purified RGCs, the glial release of TNFα in simulated ischemic conditions could explain the observed neuroprotection by TNFα- and TNF-R1-neutralizing antibodies.

The results obtained with retinal cell cultures clearly indicate that TNFα is not toxic to RGCs when the cells are surrounded by other retinal cell types and are prepared in a normoxic context. This result may explain why TNFα is not toxic in vivo under normoxic conditions and has even been reported to provide neuroprotection.[13] In mixed retinal cell culture, the lack of TNFα-induced RGC toxicity despite TNF-R1 expression can be explained by activation of a signaling pathway that is likely to involve NF-κB activation (discussed later). During ischemia, this survival pathway seems to be poorly activated, as TNFα secreted by glial cells is responsible for approximately 20% of adult RGC death (Fig. 2B). In our cell cultures, TNFα- and TNF-R1-neutralizing antibodies indeed led to this level of RGC protection. Therefore, although other mechanisms can induce RGC death,[10] our study results suggest that TNF-R1 activation could contribute to RGC death in ischemic conditions. This conclusion is in agreement with the reported TNF-R1 toxicity of retinal cells after increased intraocular pressure, leading to in vivo retinal ischemia.[12]

The additional work performed on isolated RGCs highlights an unexpected property of these cells. We have demonstrated the very high sensitivity of these cells to TNFα, more than 65% of the cells died when TNFα were added to normoxic cultures (Fig. 3A). Selective TNFα receptor R1 activation is supported, as protection also occurs when TNF-R1-neutralizing antibody is added along with TNFα. This TNFα-mediated toxicity implies an absence of a death-inhibition pathway in purified RGCs. This pathway recovered when retinal CM was added to the purified RGC culture. When Müller glial CM was added to RGCs exposed to TNFα, the protection obtained indicated that a major contributing factor in RGC survival arises from other retinal cells.

## NF-κB and c-FLIP Induction in RGCs

TNFα is often considered a poor inducer of cell death, unless accompanied by inhibitors of new RNAs or protein synthesis.[22,24] TNFα alone, however, has been shown to induce apoptotic cell death in cells that lack the transcription factor NF-κB.[25] Studies investigating the different signaling pathways activated by TNFα provide strong evidence of the significant antiapoptotic function of NF-κB.[26] A recent study by Micheau and Tschopp[14] demonstrated that two complexes can form after trimerization of TNF-R1 in response to TNFα activation. These complexes are mutually exclusive, as complex I leads to NF-κB activation and complex II recruits caspases 8/10. In this model, complex II mediates apoptosis only when complex I-mediated NF-κB activation is insufficient.[27,28] Further evidence for such involvement of complex I in our system was illustrated by RGC death observed when the antioxidant BHA was added to normoxic mixed cultures. This phenolic antioxidant is known to suppress, or at least attenuate, NF-κB activation.[23,29] As a consequence of NF-κB inactivation, RGC death was induced and it was further stimulated by TNFα application. The NF-κB implication in the prevention of TNFα-induced cell death is further supported by the NF-κB translocation in purified RGCs by retinal CM. These results differ substantially from those obtained recently using the same experimental conditions with RPE cells. These cells have been shown to remain resistant to TNFα-induced cell death, even after NF-κB activation is specifically blocked.[15]

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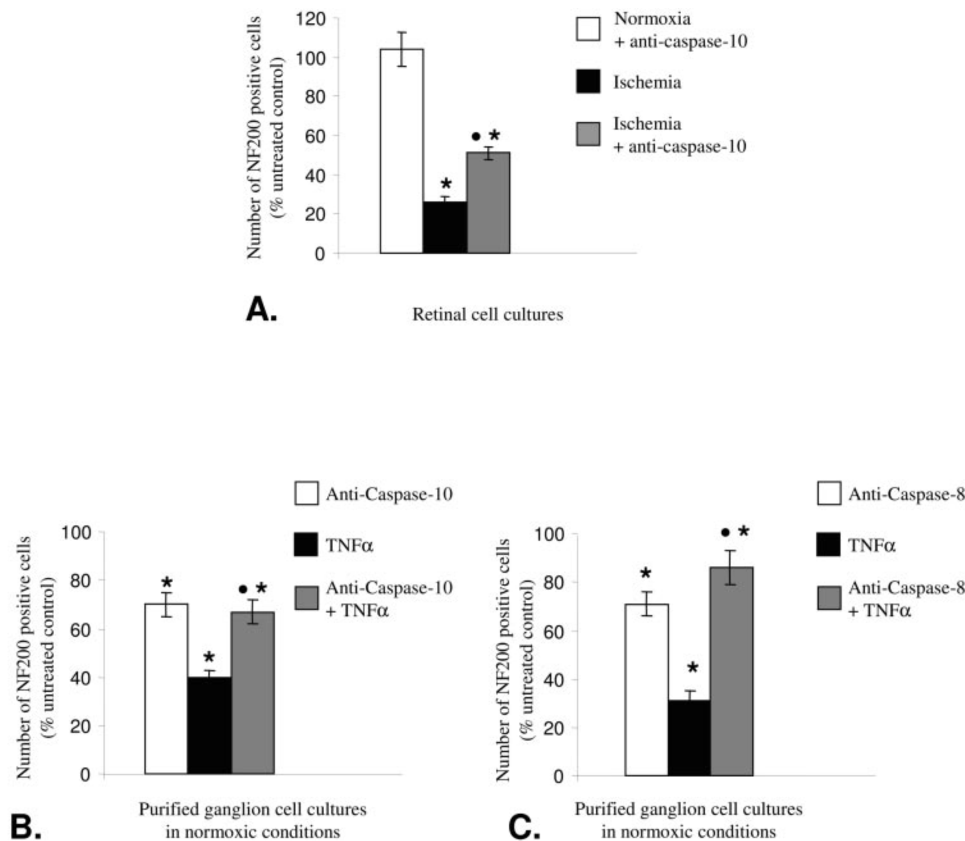


**FIGURE 6.** RGC neuroprotection by caspase-10 and -8 inhibitors in mixed retinal cell cultures and pure RGC cultures. (**A**) Retinal cell cultures were incubated in normoxic or ischemic conditions with 3 mM of caspase-10 inhibitor. RGC survival was assessed by quantitative analysis of NF200-positive cells. ANOVA and Newman-Keuls multiple-comparisons test (*$P < 0.05$, compared with untreated control cultures; •*$P < 0.05$, compared with ischemic cultures). (**B**, **C**) Purified RGC cultures were incubated in normoxic conditions with 200 ng/mL TNFα or with 200 ng/mL TNFα and 3 mM caspase-10 inhibitor (**B**) or caspase-8 inhibitor (**C**). After 10 days of culture, NF200-positive cells were assessed. ANOVA and Newman-Keuls multiple-comparisons test (*$P < 0.05$, compared with untreated control cultures; •*$P < 0.05$, compared with TNFα-treated cultures).

In the model of Micheau and Tschopp,[14] the caspase-8 homologue c-FLIP is considered as a key regulator for survival. When complex NF-κB activation is sufficient, adequate c-FLIP is expressed to inhibit caspases 8 and -10 from complex II. We demonstrated that purified RGCs were unable to synthesize the c-FLIP mRNA and protein unless they were incubated in the presence of retinal CM. The amount of c-FLIP present in the initial purified RGCs slowly disappeared and mRNA was no longer present within the cells after 10 days of culture. After 5 days of culture, TNFα accelerated further the decrease in c-FLIP expression.

Given that NF-κB regulates the expression of a wide range of proinflammatory mediators[30] and because suppression of apoptosis usually promotes inflammation,[3] the absence of c-FLIP-mediated survival in RGCs could be related to the specific immune status of the retina that minimizes inflammation.[31] Alternatively, this absence may be due to modifications in the lipid rafts on RGC membranes when these cells are grown alone. Blocking formation of such lipid rafts inhibits ubiquitination of TNF-R1 (and RIP), blocks NF-κB activation, and may inhibit the synthesis of survival factors such as c-FLIP, resulting in an increased sensitivity to TNFα-mediated apoptosis.[32] More investigations are needed to determine the precise mechanism of c-FLIP synthesis blockage in RGCs.

The critical role of the specific balance between c-FLIP and caspase-8/10 is illustrated by the reduced RGC death rate observed when caspase-8/10 inhibitors were added to the cultures. This result is in agreement with those reported in other studies in which inhibition of caspase-8 significantly improved RGC survival.[33–35] This result also supports recent work performed in the context of focal cerebral ischemia in which anti-caspase therapy provided neuroprotection.[36] This more specific anti-caspase approach seems preferable to anti-TNFα therapies that increase the risk of development of specific diseases.[37] Recent advances require the fusion of anti-caspase molecules with a protein transduction domain (TAT) from human immunodeficiency virus-I. This HIV-TAT protein contains an 11-amino-acid transduction domain that is able, when linked to a protein, to carry it across cellular membranes. Given that TAT-Bcl-XL has been proven to provide partial protection to RGCs from apoptosis,[38] we are currently investigating the antiapoptotic properties of the TAT-FLIP molecule, both in vitro and in vivo, when RGCs are exposed to ischemic conditions.

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## *References*


1. Saito K, Suyama K, Nishida K, Sei Y, Basile AS. Early increases in TNF-alpha, IL-6 and IL-1 beta levels following transient cerebral ischemia in gerbil brain. *Neurosci Lett*. 1996;206:149–152.
2. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol*. 2003;3:745–756.
3. Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol*. 2001;11:372–377.
4. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ*. 2003;10:45–65.
5. de Kozak Y, Naud MC, Bellot J, Faure JP, Hicks D. Differential tumor necrosis factor expression by resident retinal cells from experimental uveitis-susceptible and -resistant rat strains. *J Neuroimmunol*. 1994;55:1–9.
6. de Vos AF, van Haren MA, Verhagen C, Hoekzema R, Kijlstra A. Kinetics of intraocular tumor necrosis factor and interleukin-6 in


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endotoxin-induced uveitis in the rat. *Invest Ophthalmol Vis Sci*. 1994;35:1100–1106.

7. de Kozak Y, Cotinet A, Goureau O, Hicks D, Thillaye-Goldenberg B. Tumor necrosis factor and nitric oxide production by resident retinal glial cells from rats presenting hereditary retinal degeneration. *Ocul Immunol Inflamm*. 1997;5:85–94.
8. Spranger J, Meyer-Schwickerath R, Klein M, Schatz H, Pfeiffer A. TNF-alpha level in the vitreous body: increase in neovascular eye diseases and proliferative diabetic retinopathy [in German]. *Med Klin* (*Munich*). 1995;90:134–137.
9. Tezel G, Li LY, Patil RV, Wax MB. TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes. *Invest Ophthalmol Vis Sci*. 2001;42:1787–1794.
10. Tezel G, Wax MB. Increased production of tumor necrosis factor-alpha by glial cells exposed to simulated ischemia or elevated hydrostatic pressure induces apoptosis in cocultured retinal ganglion cells. *J Neurosci*. 2000;20:8693–8700.
11. Yuan L, Neufeld AH. Tumor necrosis factor-alpha: a potentially neurodestructive cytokine produced by glia in the human glaucomatous optic nerve head. *Glia*. 2000;32:42–50.
12. Fontaine V, Mohand-Said S, Hanoteau N, Fuchs C, Pfizenmaier K, Eisel U. Neurodegenerative and neuroprotective effects of tumor Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. *J Neurosci.* 2002;22(RC216):1–7.
13. Diem R, Meyer R, Weishaupt JH, Bahr M. Reduction of potassium currents and phosphatidylinositol 3-kinase-dependent AKT phosphorylation by tumor necrosis factor-(alpha) rescues axotomized retinal ganglion cells from retrograde cell death in vivo. *J Neurosci*. 2001;21:2058–2066.
14. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell*. 2003;114: 181–190.
15. Yang P, McKay BS, Allen JB, Jaffe GJ. Effect of NF-kappa B inhibition on TNF-alpha-induced apoptosis in human RPE cells. *Invest Ophthalmol Vis Sci*. 2004;45:2438–2446.
16. Mauch DH, Nagler K, Schumacher S, et al. CNS synaptogenesis promoted by glia-derived cholesterol. *Science*. 2001;294:1354–1357.
17. Beattie EC, Stellwagen D, Morishita W, et al. Control of synaptic strength by glial TNFalpha. *Science*. 2002;295:2282–2285.
18. Barres BA, Silverstein BE, Corey DP, Chun LL. Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. *Neuron*. 1988;1:791–803.
19. Luo X, Lambrou GN, Sahel JA, Hicks D. Hypoglycemia induces general neuronal death, whereas hypoxia and glutamate transport blockade lead to selective retinal ganglion cell death in vitro. *Invest Ophthalmol Vis Sci*. 2001;42:2695–2705.
20. Guidry C, Bradley KM, King JL. Tractional force generation by human Müller cells: growth factor responsiveness and integrin receptor involvement. *Invest Ophthalmol Vis Sci*. 2003;44:1355–1363.
21. McGillem GS, Guidry C, Dacheux RF. Antigenic changes of rabbit retinal Müller cells in culture. *Invest Ophthalmol Vis Sci*. 1998; 39:1453–1461.
22. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science*. 1996; 274:787–789.
23. Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L. Redox regulation of NF-kappa B activation. *Free Radic Biol Med*. 1997; 22:1115–1126.
24. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science*. 1996;274:782–784.
25. Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol*. 2002;3:221–227.
26. Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. *Cell*. 1996;87:565–576.
27. Kucharczak J, Simmons MJ, Fan Y, Gelinas C. To be, or not to be: NF-kappaB is the answer: role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene*. 2003;22:8961–8982.
28. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell*. 2004;116:205–219.
29. Faure V, Courtois Y, Goureau O. Tyrosine kinase inhibitors and antioxidants modulate NF-kappaB and NOS-II induction in retinal epithelial cells. *Am J Physiol*. 1998;275:C208–C215.
30. D'Acquisto F, May MJ, Ghosh S. Inhibition of nuclear factor kappa B (NF-κB): an emerging theme in anti-inflammatory therapies. *Mol Intervent*. 2002;2:22–35.
31. Streilein JW. Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat Rev Immunol*. 2003;3:879–889.
32. Legler DF, Micheau O, Doucey MA, Tschopp J, Bron C. Recruitment of TNF receptor 1 to lipid rafts is essential for TNFalpha-mediated NF-kappaB activation. *Immunity*. 2003;18:655–664.
33. Lin Y, Devin A, Rodriguez Y, Liu ZG. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev*. 1999;13:2514–2526.
34. Oshitari T, Adachi-Usami E. The effect of caspase inhibitors and neurotrophic factors on damaged retinal ganglion cells. *Neuroreport*. 2003;14:289–292.
35. Weishaupt JH, Diem R, Kermer P, Krajewski S, Reed JC, Bahr M. Contribution of caspase-8 to apoptosis of axotomized rat retinal ganglion cells in vivo. *Neurobiol Dis*. 2003;13:124–135.
36. Onteniente B, Couriaud C, Braudeau J, Benchoua A, Guegan C. The mechanisms of cell death in focal cerebral ischemia highlight neuroprotective perspectives by anti-caspase therapy. *Biochem Pharmacol*. 2003;66:1643–1649.
37. Gardam MA, Keystone EC, Menzies R, et al. Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. *Lancet Infect Dis*. 2003;3:148–155.
38. Dietz GP, Kilic E, Bahr M. Inhibition of neuronal apoptosis in vitro and in vivo using TAT-mediated protein transduction. *Mol Cell Neurosci*. 2002;21:29–37.

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