**Interleukin-10 Receptor Signaling through STAT-3 Regulates the Apoptosis of Retinal Ganglion Cells in Response to Stress**

Zachary S. Boyd,1 Aleksei Kiatchko,1 Junjie Yang,2 Neeraj Agarwal,3 Martin B. Wax,2,4 and Rajkumar V. Patil1,5

**PURPOSE.** Interleukin (IL)-10 has recently been shown to promote survival of neurons and glia. The purpose of this report is to investigate whether IL-10 has any role in protecting retinal ganglion cells (RGCs) from death under conditions in which growth factors are removed, or in which oxidative stress is present. Signal transduction pathways that activate IL-10 signaling in RGCs were studied in both stress conditions.

**METHODS.** Effects of various interleukins on the viability of the RGC cell line was determined, and apoptotic cells were quantified. Immunoblot analysis was preformed to identify the IL-10 receptor (IL-10R) and phosphorylated or nonphosphorylated Akt and STAT-3 proteins in RGC extracts. Immunohistochemistry was performed on the rat retinal sections to identify native IL-10R.

**RESULTS.** Apoptosis of RGCs in the absence of growth factors with or without dexamethasone (1 mM) occurred in 68.5% ± 3.4% and 53.4% ± 2.6% of cells, respectively, after 96 hours. Addition of IL-10 at a concentration of 50 ng/mL significantly reduced the apoptotic population of RGCs to 28.2% ± 2.3% in the absence of growth factors with dexamethasone and to 31% ± 2.7% in the absence of growth factors alone. RGCs as well as native retina expressed functional IL-10R as determined by immunoblot analysis and by the ability of IL-10 to phosphorylate Stat-3. However, IL-10 failed to phosphorylate Akt in these cells.

**CONCLUSIONS.** IL-10 caused a 59% and 42% reduction in the apoptotic population of serum-deprived cells with and without dexamethasone treatment, respectively. These observations establish that activation of IL-10R promotes survival of RGCs and this survival-promoting activity is due to IL-10 signaling through the Stat-3 pathway, which inhibits the cell death and not through the Akt cell survival pathway. (Invest Ophthalmol Vis Sci. 2005;44:5206–5211) DOI:10.1167/iovs.03-0534

The cytokine IL-10 has been shown to improve neurologic outcome after central nervous system (CNS) injury,1,2 promote growth and survival of numerous cancer cells,3 and render neurons in culture less vulnerable to ischemic and EAA-mediated damage.4 Primary neuronal cortical cultures derived from IL-10 knockout mice, were more susceptible to both excitotoxicity and combined oxygen-glucose deprivation compared with cell cultures from wild-type mice.4 Moreover, when added to the culture medium, recombinant murine IL-10 exerted a concentration-dependent prevention of neuronal death induced by excitotoxicity in both cortical and cerebellar granule cell cultures taken from either strain. IL-10 also provided survival signals for nonactivated cells,5,6 which were mediated by either the proliferative or antiapoptotic activity of IL-10. IL-10 directly increased survival of both cortical and cerebellar granule neurons,7 astrocytes,8 and oligodendrocytes9 and saved microglial cells from apoptosis by inhibiting the mitochondria-initiated apoptotic cell death pathway.10 These observations demonstrate that IL-10 has potential neuroprotective role in regulating neuronal cell survival.

In vitro studies using primary cocultures of retinal ganglion cells (RGCs) and glial cells have provided direct evidence that elevated pressure or ischemia, which are two prominent stress factors identified in glaucomatous eyes, can initiate apoptotic cell death in RGCs, largely through TNF-α secreted by reactive glial cells in response to these stressors.5 Furthermore, inhibiting the activity of TNF-α attenuates retinal ganglion cell death in these cultures. The purpose of this study was to test the hypothesis that anti-inflammatory effects of IL-10 may improve neurologic outcome of RGCs after the specific neuronal insult of serum deprivation or oxidative stress. Our results provide new evidence that IL-10 promotes the survival of serum-deprived (growth factor withdrawal) as well as dexamethasone-treated (which promotes oxidative stress and proapoptosis environment) RGCs in culture. In addition, we identified the presence of IL-10 receptor (IL-10R) protein in these cells and demonstrated that activation of IL-10R inhibits the apoptosis of RGCs by stimulating the STAT-3 but not the PI3-kinase/Akt signaling pathway.

**MATERIALS AND METHODS**

**Cell Culture**

The rat RGC cell line transformed with E1A virus was used in this study. These cells express RGC-specific markers such as Thy-1 and Bm-3.10 Further, these cells do not express glial fibrillary acidic protein (GFAP; a positive marker for Müller cells), HPC-1/syntaxin (α marker for amacrine cells), and 8A1 (a marker for horizontal cells) suggesting they represent a genuine RGC type. In addition, as seen with the primary RGC cell type, these cells were also positive for the expression of neurotrophins and their cognate receptors.
Cell Survival Assay

To determine the effect of interleukins on the viability of RGCs after serum deprivation, an equal number of RGCs (5 × 10^5) were seeded into 96-well plates in either serum-free (SF) Dulbecco’s-modified Eagle’s medium (DMEM; Mediatech Inc., Herndon, VA) or in DMEM containing 10% FBS (complete DMEM) and maintained at 37°C in a humidified CO2 incubator. After 12 hours, cells on SF medium were treated with the anti-inflammatory cytokines IL-2, IL-4, and IL-10 (four wells each at 50 ng/mL) diluted in SF DMEM. Cellular viability after cytokine treatment up to 120 hours was observed using a formazan assay kit (CellTiter 96; Promega, Madison, WI). Viable cells metabolize the MTT tetrazolium salt in the dye solution into a formazan product. Cells were incubated with dye solution for 4 hours, and then treated with solubilization-stop solution. Spectrophotometric absorption at 590 nm was measured with a plate reader (Multiskan 340MK2, Titer-Tek, Huntsville, AL). The effect of cytokines on viability was reported as percentage of cells surviving, and was defined as the average percentage of measured cellular viability in SF medium in comparison to that of cells maintained in complete DMEM.

Western Blot Analysis

The RGCs were harvested and lysed in ice-cold lysis buffer containing 2 mM HEPES, 2 mM EDTA (pH 7.4) and protease inhibitors (50 μM phenylmethylsulfonyl fluoride and 1 μg/mL each of aprotonin, antipain, leupeptin, and pepstatin A) for 5 minutes at 4°C. After centrifugation at 1000g for 5 minutes, the supernatant (50 μg protein) was used for Western blot analysis. Samples were separated by electrophoresis in 12% SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) using a semidyey transfer system (Bio-Rad, Hercules, CA). After transfer, membranes were blocked in a buffer (50 mM Tris-HCl, 154 mM NaCl, 2 mM HEPES, 2 mM EDTA (pH 7.4) and protease inhibitors (50 μM phenylmethylsulfonyl fluoride and 1 μg/mL each of aprotonin, antipain, leupeptin, and pepstatin A) for 5 minutes at 4°C). After centrifugation at 1000g for 5 minutes, the supernatant (50 μg protein) was used for Western blot analysis. Samples were separated by electrophoresis in 12% SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The IL-10R antibodies directed against either the carboxyl (M-20) or amino (K-20) terminus of the IL-10R protein (Santa Cruz Biotechnology. Santa Cruz, CA) were used to detect the IL-10R protein in the RGC extract. Phospho-Akt (Ser473) and phospho-STAT-3 (Tyr705) antibodies, both from Cell Signaling Technology (Beverly, MA), were used to detect phosphorylated Akt and STAT-3 proteins, respectively.

Immunohistochemistry

For immunostaining of IL-10 receptors in rat retina, sections from normal eyes were deparaffinized, rehydrated, and pretreated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) to decrease endogenous peroxidase activity. A polyclonal antibody (M-20) directed against the carboxyl terminus of the IL-10R protein (Santa Cruz Biotechnology) was used to detect the IL-10R protein in the retinal sections. The immunostaining was visualized with reagents purchased from Vector Laboratories (Burlingame, CA). The biotinylated secondary antibody was incubated with the sections for 30 minutes, washed with PBS solution containing 0.1% bovine serum albumin, and reacted with streptavidin-horseradish peroxidase conjugated for 30 minutes. After several washes, color was developed by incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO) as a cosubstrate, for 5 to 7 minutes. Sections were counterstained with hematoxylin and mounted (Permount; Fisher Scientific, Pittsburgh, PA). For a negative control, preimmune rabbit serum (Sigma-Aldrich) was used to replace the primary antibodies. Slides were examined by microscope (Nikon, Tokyo, Japan), and images were recorded with a digital camera (Optronics, Goleta, CA).

Measurement of Apoptosis by Flow Cytometry

An apoptotic detection kit (Molecular Probes, Eugene, OR) was used to quantify apoptotic cells after their respective treatments. The kit incorporates a proprietary dye (YO-PRO-1), which selectively passes through the plasma membranes of apoptotic cells and stains them with green fluorescence due to characteristic fragmentation of the chromatin within the nucleus. Necrotic cells are stained fluorescent red with propidium iodide. Briefly, cells were incubated in SF medium in the presence or the absence of IL-10 and dexamethasone, trypsinized, washed with PBS, and stained according to the manufacturer’s protocol. Cultured cells in the presence of 10% serum served as the control. Cells were analyzed by flow cytometry (FACScan with CellQuest Software; BD Biosciences, Lincoln Park, NJ) with 488-nm excitation, and the percentage of apoptotic cells per 100,000 was established for each treatment group.

Results

Effect of Cytokines on the Survival of RGCs

Cellular viability after cytokine treatment was determined using a formazan assay. As is shown in Figure 1A, IL-2 and -4 had no effect on the MTT metabolizing activity (i.e., cell viability) of the RGCs, whereas, treatment of IL-10 significantly increased the cell viability of RGCs in the absence of growth factors. The effect of IL-10 on the RGC cell viability was time (Fig. 1B) and dose dependent (Fig. 1C); the optimal effect was at 96 hours at a concentration of 50 ng/mL. These results suggest that IL-10 plays a critical role in the survival of RGCs in culture.

Because data derived from the MTT assay is indicative of viable cells only, it was not clear whether the increase in MTT conversion was a result of IL-10-induced cell proliferation or inhibition of cell death. To clarify this question, we used flow cytometry for direct quantitation of the number of apoptotic cells after IL-10 treatment. RGCs were washed with DMEM and cells were plated for 96 hours in SF medium alone, in SF medium containing dexamethasone (1 μM), inn SF medium containing IL-10 (50 ng/mL), or in SF medium containing both dexamethasone and IL-10. Cells plated in medium containing 10% FBS exhibited the absence of IL-10 and dexamethasone were used as a control in these experiments. Dexamethasone has been shown to promote oxidative stress by increasing hydrogen peroxide production in a variety of cell types including vascular endothelial cells and is a direct inducer of apoptotic cell death by suppressing NF-κB in hippocampal cells. The percentage of apoptotic cells in the presence of IL-10 in either SF medium (31% ± 1%, n = 4) or SF medium supplemented with dexamethasone (28.2% ± 2.2%, n = 4) was similar to control medium (31.5% ± 3%, n = 4; Fig. 2). Whereas, the percentage of apoptotic cells in the SF medium (53.4% ± 3.4%, n = 4) and SF medium supplemented with dexamethasone (68.4% ± 4.7%, n = 4) was significantly elevated in contrast IL-10 treated cells. These findings suggest that IL-10 induces the survival of RGCs by inhibiting the apoptotic cell death, not by promoting cellular proliferation. The cells were able to proliferate in the presence of 10% FBS, as the number of cells was increased by approximately fourfold (n = 3; data not shown). These results are consistent with those of others who found that IL-10 did not enhance the proliferation of murine microglial cells.

IL-10 Receptor Protein in Rat Retina and Cultured RGCs

To characterize the IL-10-mediated signal-transducing pathway in RGCs, we first sought to learn whether these cells express the IL-10 receptor. Detection of IL-10R protein in the RGC cell lysates and retinal sections were assessed by Western blot analysis and immunohistochemistry, respectively (Fig. 3),
using IL-10R antibodies. RGCs were harvested and lysed as described. The lysate (50 μg protein) was used for Western blot analysis. An immunoreactive band of 90 kDa was detected in the RGC lysates (Fig. 3A). This band was higher than the expected IL-10R band based on its actual molecular mass of 63 kDa. As a result of an extensive glycosylation of the receptor, the electrophoretic mobility of native IL-10R has been reported slower than predicted from the amino acid sequence.\textsuperscript{14,15} Similar findings are reported from other cell types that express IL-10R protein.\textsuperscript{8,16} As seen in Figure 3B, immunohistochemical staining of rat retinal sections using IL-10R antibody demonstrated constitutive expression of IL-10R protein in the inner nuclear layer (INL) and ganglion cell layer (GCL).
factor (IGF)-1 is a potent inducer of this pathway, we first confirmed the Akt activation by IGF-1 in RGCs. In these experiments, RGCs were stimulated with IGF-1 (100 ng/mL; for 2 and 10 minutes), cells were lysed, and the phosphorylated Akt was visualized using phospho-Akt antibody. Akt was phosphorylated after treatment with IGF-1 (Fig. 4, lanes 4, 5). As expected, a low level of Akt phosphorylation was detected in control cells that were not treated with IGF-1 (lane 1). These data establish that Akt phosphorylation can easily be detected after the activation of IGF receptor in RGCs.

Parallel experiments were conducted to determine whether IL-10 activates Akt in RGCs. Cells were treated with IL-10 (50 ng/mL), as with IGF-1, and lysed. Proteins were separated on 10% SDS-PAGE gels, transferred to PVDF membranes and blotted with an anti-phospho-Akt antibody that detects phosphorylation of Ser473. Because insulin-like growth factor (IGF)-1 is a potent inducer of this pathway, we first confirmed the Akt activation by IGF-1 in RGCs. In these experiments, RGCs were stimulated with IGF-1 (100 ng/mL; for 2 and 10 minutes), cells were lysed, and the phosphorylated Akt was visualized using phospho-Akt antibody. Akt was phosphorylated after treatment with IGF-1 (Fig. 4, lanes 4, 5). As expected, a low level of Akt phosphorylation was detected in control cells that were not treated with IGF-1 (lane 1). These data establish that Akt phosphorylation can easily be detected after the activation of IGF receptor in RGCs.

Phosphorylation of Akt by IGF-1 and IL-10 in RGCs

IL-10 has recently been reported to promote survival of astrocytes by activating phosphatidylinositol (PI)3-kinase and Akt. Phosphorylation of Ser473 amino acid residue is essential for protein kinase activity of Akt. Because insulin-like growth factor (IGF)-1 is a potent inducer of this pathway, we first confirmed the Akt activation by IGF-1 in RGCs. In these experiments, RGCs were stimulated with IGF-1 (100 ng/mL; for 2 and 10 minutes), cells were lysed, and the phosphorylated Akt was visualized using phospho-Akt antibody. Akt was phosphorylated after treatment with IGF-1 (Fig. 4, lanes 4, 5). As expected, a low level of Akt phosphorylation was detected in control cells that were not treated with IGF-1 (lane 1). These data establish that Akt phosphorylation can easily be detected after the activation of IGF receptor in RGCs.

Parallel experiments were conducted to determine whether IL-10 activates Akt in RGCs. Cells were treated with IL-10 (50 ng/mL), as with IGF-1, and lysed. Proteins were separated on 10% SDS-PAGE gels, transferred to PVDF membranes and blotted with an anti-phospho-Akt antibody that detects phosphorylation of Ser473. There was no change in the phosphorylation of Akt after treatments with IL-10 for 2 and 10 minutes (Fig. 4, lanes 2 and 3). There was no change in the phosphorylation of Akt up to 40 minutes after IL-10 treatment (data not shown). Lanes 6 and 7 were loaded with 20 μL of nonphosphorylated and phosphorylated Akt cell extracts from Jurkat cells as negative and positive controls, respectively (Cell Signaling Technologies). To ensure equal protein loading, Western blots were probed with antibodies to unphosphorylated Akt (Fig. 4, bottom panel). These results establish that IL-10 does not activate Akt in RGCs, suggesting that IL-10 may induce other signaling events in these cells that mediate cell survival effects.

IL-10 Phosphorylates STAT-3 in RGC

STAT-3 is a key-signaling molecule for many cytokines and growth factor receptors. To further understand the signaling events underlying IL-10-induced survival of RGCs, we studied the role of the STAT pathway. The RGCs were deprived of serum for 12 hours and treated with and without IL-10 (50 ng/mL) for various times, and cell extracts were prepared. Equal amounts of protein from the control and each treatment were analyzed by Western blot for tyrosine (Tyr705) phosphorylation of STAT-3 using phospho-Stat3 antibody. A significant increase in tyrosine phosphorylation of STAT-3 was observed at 10 minutes (Fig. 5, lane 3). Phosphorylation of STAT-3 was seen as early as 1 minute (lane 2) and as late as 30 minutes (data not shown) after IL-10 treatment. No phosphorylation of STAT-1 was observed after the treatment with IL-10 (up to 30 minutes; data not shown).

Because ciliary neurotrophic factor (CNTF) is a potent inducer of this pathway, we confirmed the STAT-3 activation by CNTF in RGCs. In these experiments, RGCs were stimulated with CNTF (100 ng/mL; 2 minutes), cells were lysed and the phosphorylated STAT-3 was visualized using phospho-Stat3 (Tyr705) antibody. STAT-3 was phosphorylated in RGCs when treated with CNTF (Fig. 5, lane 4). STAT-3 phosphorylation was not detected in control cells that were not treated with either IL-10 or CNTF (lane 1). To ensure equal protein loading, Western blots were probed with antibodies to nonphosphorylated STAT-3 (Fig. 5, bottom panel). These results establish that stimulation of IL-10 receptors in RGCs activate the phosphorylation of STAT-3.

DISCUSSION

IL-10 plays an essential role in mediating inflammatory processes and cell survival in the immune system and brain. We have extended these findings by showing that IL-10 promotes the survival of serum-deprived RGCs or RGCs experiencing oxidative stress due to dexamethasone treatment. Our findings confirm the antiapoptotic protection by IL-10 of cultured rat RGCs and demonstrate that these cells express IL-10R protein. Further, we observed that stimulation of IL-10R by its ligand leads to tyrosine phosphorylation of STAT-3, but it does not activate the Akt pathway in cultured rat RGCs subjected to growth-factor deprivation. In contrast, IGF-1, used as a positive control increases the phosphorylation of Akt. Despite the ability of IL-10 to induce STAT-3 phosphorylation and to increase cell viability, IL-10 is unable to promote cell proliferation. Instead, the IL-10-induced survival of RGCs is caused by an increase in cell survival rather than in cell proliferation.

![Figure 4](image4.png)  
**Figure 4.** Akt was phosphorylated in retinal ganglion cells in response to IGF-1 but not IL-10 treatment. RGCs were cultured in SF medium for 12 hours and subsequently treated with IL-10 (50 ng/mL; 2 and 10 minutes) or IGF (100 ng/mL; 2 and 10 minutes). Whole lysates were separated on 10% SDS polyacrylamide gels and electrophoretically transferred to PVDF membranes. Akt activation was measured in immunoblots with a phospho-Akt antibody. To ensure equal protein loading, Western blots were probed with antibodies to unphosphorylated Akt. Similar results were obtained in three separate experiments.

![Figure 5](image5.png)  
**Figure 5.** STAT-3 was phosphorylated in RGCs in response to IL-10 treatment. RGCs were cultured in SF medium for 12 hours and subsequently treated with IL-10 (50 ng/mL; 10 minutes) or CNTF (100 ng/mL; 10 minutes). Whole lysates were separated on 10% SDS polyacrylamide gels and electrophoretically transferred to PVDF membranes. STAT-3 activation was measured by blotting with a phospho-STAT-3 antibody. To ensure equal protein loading, Western blots were probed with antibodies to unphosphorylated STAT-3. Similar results were obtained in three separate experiments.
results are consistent with others who showed that IL-10 does not enhance the proliferation of primary microglia.15 In addition, these results suggest that IL-10 promotes cell survival by a mechanism that may involve activation of STAT-3 pathways. STAT-3 is a latent transcription factor required for the anti-inflammatory action of IL-1016 and for promoting tumor survival.17 Of note, our earlier preliminary results, which have been presented in abstract form (Boyd ZS, et al. IOVS 2003; 44: ARVO EAbstract 125), using antibody specific to phospho-STAT-3 (Ser727) failed to demonstrate the activation of STAT-3. Although maximum activation of STAT-3 depends on the phosphorylation at Ser727, Tyr705 phosphorylation is obligatory to become STAT-3 active.20–22 Therefore, we repeated these studies using antibody specific to STAT-3 phosphorylated at Tyr705 in the present study. It appears that IL-10 is activating JAK/Tyk tyrosine kinase pathways in RGCs that phosphorylate STAT-3 at Tyr705 and not the MAPK or mammalian target of rapamycin (mTOR) serine kinase pathways that phosphorylate STAT-3 at Ser727.

Although IL-10 activates STAT-3 and IGF induces Akt, the biological outcome of these two pathways could be similar. Subsequent to activation, STAT-3,23–25 PI 3-kinase,26–28 and Akt26–29,30 promote survival signals in a variety of cells. For example, PI 3-kinase activity is implicated in IL-10-induced proliferation of murine mast cells31 and the survival of astrocytes.6 However, in RGCs, IL-10 did not induce phosphorylation of Akt, suggesting that PI 3-kinase activity may not be involved in IL-10-mediated survival. Despite the supporting evidence for an interaction between the IL-10 and PI 3-kinase pathways, the mechanism of this association in RGCs is not clear.

IL-10 is known as an inhibitor of the synthesis of inflammatory cytokines, including TNF-α and IL-1.2,13,32,33 Recent reports have revealed increased levels of TNF-α in glaucomatous retinas obtained at autopsy34 and in human glial cells exposed to stimulated ischemia or elevated pressure35 and higher levels of serum IL-10 in patients with glaucoma.36 These observations provide evidence that regulation of immunogenic capacity of retinal neuronal cells by cytokines may have a role during the neurodegeneration process in patients with glaucoma. Our studies strongly support a survival role of IL-10 for serum-deprived or dexamethasone-treated RGCs in culture and suggest that STAT-3 may be responsible for mediating the anti-apoptotic effects of IL-10. An understanding of molecular pathways responsible for IL-10-mediated RGC survival may yield novel therapies to regulate RGC death in glaucoma. In contrast, the potential for cytokine-based therapy for glaucoma is not yet clear. There is a threat of autoimmunity after any attempt of protective vaccination by IL-10.36 However, there is accumulating evidence for neuroprotection of RGCs in animal models after immunization with self-antigens. For example, survival of RGCs after axonal crush injury was enhanced by vaccination with self-antigen,39 and systemic administration of IL-10 reduced rat brain injury after focal stroke.40 In addition, a recent report suggests that vaccination with Cop-1, a synthetic copolymer, can protect RGCs against death in a rat glaucoma model.41 Thus, whether IL-10 will ultimately be clinically useful in protecting RGCs depends on future studies with in vivo administration of IL-10 in a animal model of glaucoma.

References


