

MOLECULAR AND SYNAPTIC MECHANISMS

Tumor necrosis factor-alpha mediates activation of NF- κ B and JNK signaling cascades in retinal ganglion cells and astrocytes in opposite ways

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Abstract

Tumor necrosis factor-alpha (TNF) is an important mediator of the innate immune response in the retina. TNF can activate various signaling cascades, including NF- κ B, nuclear factor kappa B (NF- κ B) and c-Jun N-terminal kinase (JNK) pathways. The harmful role of these pathways, as well as of TNF, has previously been shown in several retinal neurodegenerative conditions including glaucoma and retinal ischemia. However, TNF and TNF-regulated signaling cascades are capable not only of mediating neurotoxicity, but of being protective. We performed this study to delineate the beneficial and detrimental effects of TNF signaling in the retina. To this end, we used TNF-treated primary retinal ganglion cell (RGC) and astrocyte cultures. Levels of expression of NF- κ B subunits in RGCs and astrocytes were evaluated by quantitative RT-PCR (qRT-PCR) and Western blot (WB) analysis. NF- κ B and JNK activity in TNF-treated cells was determined in a time-dependent manner using ELISA and WB. Gene expression in TNF-treated astrocytes was measured by qRT-PCR. We found that NF- κ B family members were present in RGCs and astrocytes at the mRNA and protein levels. RGCs failed to activate NF- κ B in the presence of TNF, a phenomenon that was associated with sustained JNK activation and RGC death. However, TNF initiated the activation of NF- κ B and mediated transient JNK activation in astrocytes. These events were associated with glial survival and increased expression of neurotoxic pro-inflammatory factors. Our findings suggest that, in the presence of TNF, NF- κ B and JNK signaling cascades are activated in opposite ways in RGCs and astrocytes. These events can directly and indirectly facilitate RGC death.

Introduction

Tumor necrosis factor-alpha (TNF) is an inflammatory cytokine that is involved in numerous physiological and pathological conditions in the central nervous system (CNS) (Perry *et al.*, 2002; McCoy & Tansey, 2008). While TNF may participate in the normal development and maintenance of nervous tissue, it is an important mediator of the innate immune response in the CNS, including the retina (De Simoni & Imeri, 1998; Perry *et al.*, 2002; Berger *et al.*, 2008; McCoy & Tansey, 2008; Tezel, 2008). It has been shown that TNF plays a largely harmful role in a wide range of retinal neurodegenerative conditions, such as glaucoma and retinal ischemia, which are leading causes of adult blindness in the world (Berger *et al.*, 2008; Tezel, 2008). This was a surprising finding, because TNF can not only mediate cell death and toxicity, it can also promote cell survival (Marques-Fernandez *et al.*, 2013). Thus, understanding the molecular and cellular mechanisms of TNF-mediated toxicity in the retina is of considerable interest for creating an effective therapy against many retinal neurodegenerative diseases.

The loss of retinal ganglion cells (RGCs) is characteristic of many retinal neurodegenerative conditions including glaucoma and retinal ischemia (Berger *et al.*, 2008; Tezel, 2008). In such neurodegenerative conditions, astrocytes can facilitate RGC death (Varela & Hernandez, 1997; Dvorianchikova *et al.*, 2009; Son *et al.*, 2010; Barakat *et al.*, 2012). Astrocytes are an important type of glial cell with which RGCs are in close contact. Astrocytes in normal tissue support neurons and facilitate neuronal function (Fields & Stevens-Graham, 2002). However, astrocytes have been predominantly associated with neuronal loss in pathological conditions in the CNS (Pekny & Nilsson, 2005; Dvorianchikova *et al.*, 2009; Barakat *et al.*, 2012). It has already been shown that TNF produced and secreted by astrocytes facilitates RGC death in RGC-astrocyte co-cultures (Tezel & Wax, 2000). Thus, the pathological effects of TNF observed in the retina can be explained in part by the activation of damaging signaling cascades in RGCs directly and indirectly due to the TNF-mediated neurotoxic activity of astrocytes. It has been shown that TNF receptors are expressed in both RGCs and astrocytes (Tezel *et al.*, 2001; Tezel, 2008). It is reported that binding of TNF to the receptor can initiate both nuclear factor kappa B (NF- κ B) and c-Jun N-terminal kinase (JNK) signaling cascades in the cell (Mak & Yeh, 2002). Activation of NF- κ B and

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transient activation of JNK are associated with cellular survival, while sustained JNK activation contributes to cell death (Kamata *et al.*, 2005; Papa *et al.*, 2006). Importantly, it has been shown that impaired NF- κ B activity results in sustained JNK activation and cell death (Kamata *et al.*, 2005; Papa *et al.*, 2006). Meanwhile, activation of NF- κ B in astrocytes promotes production of neurotoxic pro-inflammatory factors, including TNF, in these cells, and it mediates RGC death (Pekny & Nilsson, 2005; Dvorianchikova *et al.*, 2009). Considering all this information, we suggested that in the presence of TNF, NF- κ B and JNK signaling cascades are activated in opposite ways in RGCs and astrocytes, and this can facilitate neuronal death.

Materials and methods

Animals

All experiments were performed in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and the Association for Research in Vision and Ophthalmology (ARVO) statement for use of animals in ophthalmic and vision research. The University of Miami Institutional Animal Care and Use Committee (IACUC) specifically approved this study. C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under standard conditions of temperature and humidity with free access to food and water and a 12-h light–dark cycle. All animals used in our experiments were adult breeding pairs, 1-month-old mice, as well as 4- to 8- and 12-day-old pups.

NF- κ B activation assay

The preparation of cellular nuclear extracts and measurement of the NF- κ B DNA-binding activity were performed with a nuclear extraction kit (Affymetrix-Panomics, Santa Clara, CA, USA) and an NF- κ B p65 ELISA kit (Affymetrix-Panomics), respectively, according to the manufacturer's protocols.

Primary cell cultures

RGCs were isolated using the two-step immunopanning protocol as previously described (Dvorianchikova *et al.*, 2012). Isolated RGCs were cultured in serum-free basal media (Neurobasal/B27 media; Life Technologies, Grand Island, NY, USA) 1 day before the experiment. We obtained primary cortical astrocytes using the 'shaking method' as previously described (Barakat *et al.*, 2012). To prepare Müller glia, we used a previously described protocol (Hauck *et al.*, 2003). Briefly, retinas from early postnatal pups (P4–8) were digested for 30 min in papain (20 U/mL), triturated and plated onto plastic dishes. Dishes were shaken after 16 h and nonattached cells were removed. Attached Müller glial cells were grown until confluency, trypsinised and used in our study. Primary cortical astrocytes and Müller glia were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies), containing 10% heat-inactivated fetal bovine serum (FBS; Life Technologies) and 1% antibiotic–antimycotic (Life Technologies). RGCs, astrocytes and Müller glia were maintained during the experiment in half DMEM and half Neurobasal media containing 0.1% FBS and 1% antibiotic–antimycotic.

Cell death assay

Prior to the experiment, RGCs and astrocytes were plated on cover slips in a 24-well plate. After treatment with TNF, necrotic and

apoptotic RGCs and astrocytes were determined using a Vybrant Apoptosis Assay Kit, according to the manufacturer's protocol (Life Technologies). Individual glasses were sampled randomly to collect a total of 10 images using a confocal microscope (Leica TSL AOB5 SP5; Leica Microsystems). The apoptotic and necrotic cells were counted using ImageJ software. The percentages of necrotic, apoptotic and living cells relative to the total number of cells was determined. The experiments were repeated at least three times.

Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cells using the Absolutely RNA Nanoprep kit (Agilent Technologies, Santa Clara, CA, USA) and reverse-transcribed with the Reverse Transcription System (Promega, Madison, WI, USA) to synthesise cDNA as previously described (Ivanov *et al.*, 2008). Quantitative RT-PCR was performed in the Rotor-Gene Q Cycler (Qiagen, Valencia, CA, USA) using the SYBR GREEN PCR MasterMix (Qiagen) and gene-specific primers (Table 1). For each gene, relative expression was calculated by comparison with a standard curve, following normalisation to the housekeeping gene, succinate dehydrogenase subunit A (*Sdha*), whose expression was used as a control.

Western blot analysis

Primary RGC and astrocyte cultures were lysed with RIPA buffer supplemented with protease inhibitor (Roche Applied Science, Indianapolis, IN, USA). Protein concentrations were assessed using the BCA kit, according to the manufacturer's protocol (Thermo Scientific, Brookfield, WI, USA). An equal amount of total protein from each sample was resolved on SDS-PAGE gradient 4–12% Bis-Tris

TABLE 1. List of PCR primers

| Gene | Oligonucleotides | |
|---------------|------------------|------------------------|
| <i>Rela</i> | Forward | GGCCTCATCCACATGAACCTT |
| | Reverse | ATCGGATGTGAGAGGACAGG |
| <i>Relb</i> | Forward | TTGGGTCCAGTGACCTCTC |
| | Reverse | CTTTGGCTGCTCTGTGATGA |
| <i>Rel</i> | Forward | CTGGAGCCTGTGACAGTGAA |
| | Reverse | GACCTGGGCATTTCTGGTAA |
| <i>Nfkb1</i> | Forward | TTTTCGACTACGCAGTGACG |
| | Reverse | GTCCAGAAGGCTCAGGTCAG |
| <i>Nfkb2</i> | Forward | GCAGATAGCCCACGTCATTT |
| | Reverse | CAGGCATGTGCAATATTTGG |
| <i>Tnf</i> | Forward | CAAAATTCGAGTGACAAGCCTG |
| | Reverse | GAGATCCATGCCGTTGGC |
| <i>Il1b</i> | Forward | GACCTTCCAGGATGAGGACA |
| | Reverse | AGGCCACAGGATTTTTGTGCG |
| <i>Il6</i> | Forward | ATGGATGCTACCAAACTGGAT |
| | Reverse | TGAAGGACTCTGGCTTTGTCT |
| <i>Ccl2</i> | Forward | AGGTCCCTGTGCTGCTTCTG |
| | Reverse | ATTTGGTTCCGATCCAGGTT |
| <i>Ccl5</i> | Forward | AGCAGCAAGTGTCCAATCT |
| | Reverse | ATTTCTTGGGTTTGTGTGC |
| <i>Cxcl10</i> | Forward | GCTGCAACTGCATCCATATC |
| | Reverse | CACTGGGTAAGGGGAGTGA |
| <i>Cybb</i> | Forward | GACTGCGGAGAGTTTGAAG |
| | Reverse | ACTGTCCACCTCCATCTTG |
| <i>Ncf1</i> | Forward | CGAGAAGAGTTCGGGAACAG |
| | Reverse | AGCCATCCAGGAGCTTATGA |
| <i>Nos2</i> | Forward | CAGAGGACCCAGAGACAAGC |
| | Reverse | TGCTGAAACATTTCTGTGC |
| <i>Sdha</i> | Forward | ACACAGACCTGGTGGAGACC |
| | Reverse | GCACAGTCAGCCTCATTCAA |

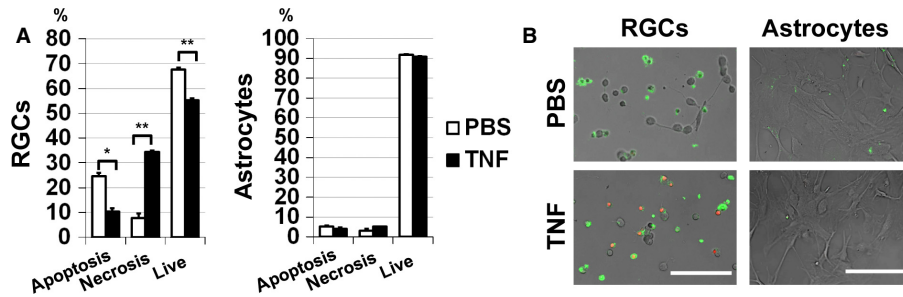


FIG. 1. TNF contributed to RGC death, while astrocytes demonstrated significant survival after TNF treatment. (A) Primary RGC and astrocyte cultures were treated with TNF (30 ng/mL) and cell death was detected after 24 h. The percentage \pm SEM of living cells as well as necrotic and apoptotic cells relative to the total number of cells was determined ($*P < 0.05$, $**P < 0.01$). The experiments were repeated at least three times. (B) Representative confocal images of Annexin V-labeled (green, a marker of apoptotic cells) and Annexin V/propidium iodide-labeled (PI, red; a marker of necrotic cells) cells in control (PBS-treated) and TNF-treated cultures were obtained using a confocal microscope. Scale bar, 100 μ m.

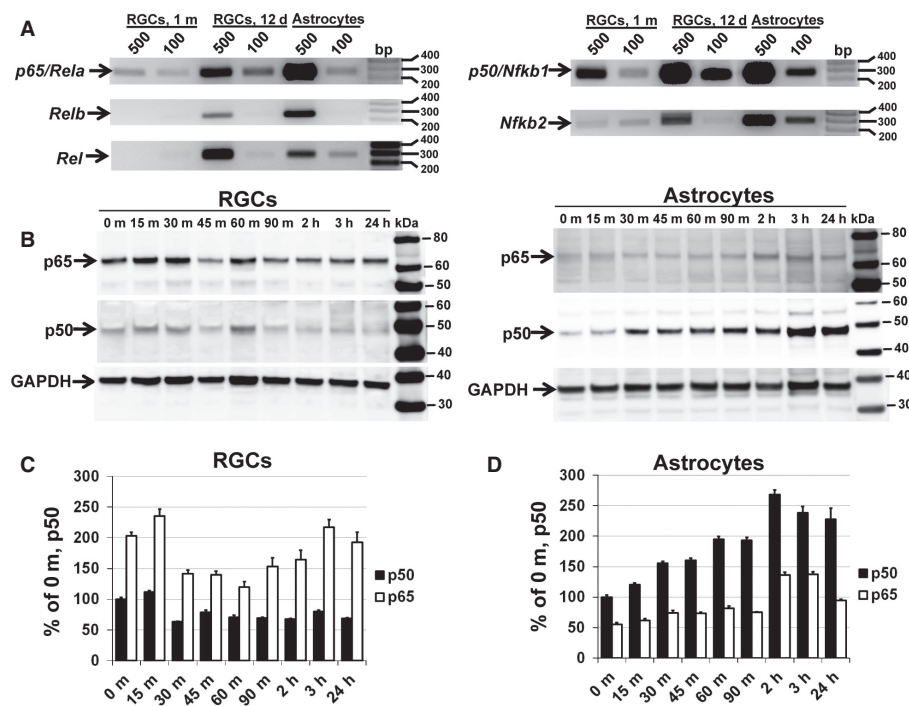


FIG. 2. NF- κ B family members were present in RGCs and astrocytes at the mRNA and protein levels. (A) Micrograph of the gel-separated PCR products from the RT-PCR test showed specific bands corresponding to mRNA for Rel α /p65, Rel β , Rel, Nf κ B1/p50 and Nf κ B2. The RNA samples were isolated from 500 astrocytes and 100 RGCs. RGCs were isolated from 1-month-old and 12-day-old mice. (B) The lysates of TNF-treated RGC and astrocyte cultures were collected in a time-dependent manner and p65 and p50 NF- κ B subunits were detected by Western blotting. Primary RGCs were isolated from 12-day-old pups; primary astrocytes were isolated from 3-day-old pups. (C and D) Quantification of NF- κ B subunit p65 and p50 levels in TNF-treated primary RGCs and astrocytes using Western blot analysis (refer to B). The p65 and p50 intensities were normalised to GAPDH. As we used the same Western blots to test p65, p50 and GAPDH levels, the normalised values are expressed as percentages of the corresponding p50 value in (C) RGCs and (D) astrocytes treated for 0 min (0 m). Values are means \pm SEM, $n = 3$.

gels and transferred to PVDF membrane (Life Technologies). Blots were blocked in 5% milk in Tris-buffered saline (TBS; pH 7.6), probed with the primary antibody against JNK (1:500; 554285, Clone G151-666, BD Biosciences/Pharmingen, San Jose, CA, USA), phospho-JNK (1:2000; 700031, Life Technologies), I κ B α (1:2500; ab32518, Abcam, Cambridge, MA, USA), p65 (1:400; sc-372, Santa Cruz Biotechnology, Dallas, TX, USA) and p50 (1:200; ab7971, Abcam) overnight, washed in 0.15% Tween20 in TBS, and incubated for 1 h with secondary antibody (1:10 000, Amersham Biosciences, NJ, USA) diluted in TBS. Anti-GAPDH antibodies (1:4000; GTX627408, GeneTex, Irvine, CA, USA) were used to control the loading. Proteins were visualised using SuperSignal chemiluminescent substrates (Thermo Scientific) and quantified using the FUJI-

FILM software. The specificity of antibodies against p65 (Rel α), p50 (Nf κ B1), I κ B α and JNK was shown previously using tissues of p65 (Rel α -), p50(Nf κ B1)-, I κ B α - and JNK-knockout animals respectively (Klement *et al.*, 1996; Kuan *et al.*, 1999; Sabapathy *et al.*, 1999; Herkenham *et al.*, 2011). The specificity of antibodies against phospho-JNK was demonstrated by the manufacturer's company (Life Technologies) using phosphopeptide immunogen.

Statistical analysis

Statistical analysis of NF- κ B activity, levels of necrotic, apoptotic and living cells, as well as changes in levels of RNA and protein expression was performed with a one-way ANOVA for multiple com-

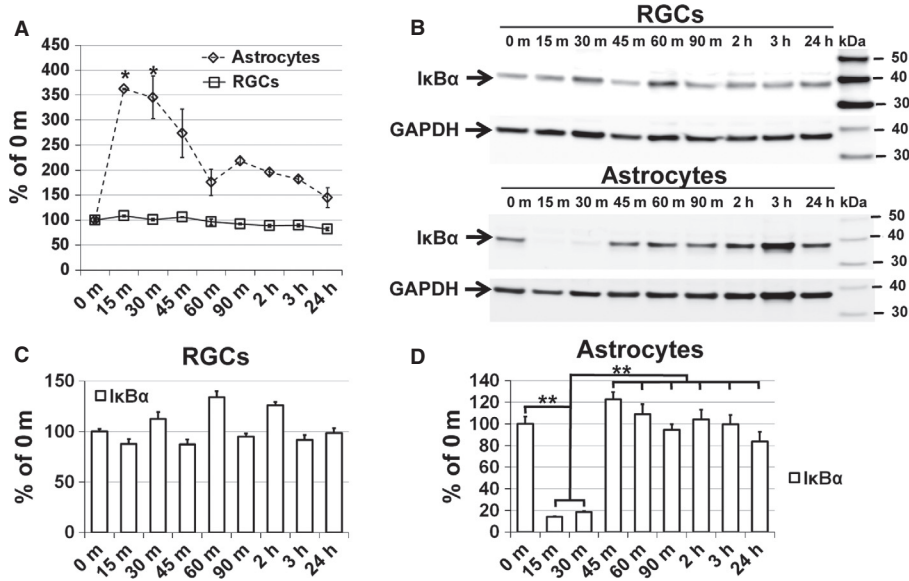


FIG. 3. RGCs failed to activate NF- κ B in the presence of TNF, while astrocyte cultures demonstrated NF- κ B activation after TNF treatment. (A) Primary RGCs and astrocytes were treated with 30 ng/mL TNF over a 24-hour time course and NF- κ B activity was measured by the NF- κ B p65 activation ELISA kit. NF- κ B activity was induced after 15 min and maintained for at least 24 h in glial cells, while NF- κ B activity in TNF-treated RGCs was not detected (9 μ g of cell nuclear extract were used). The results are expressed as percentages of the corresponding value in the cells treated for 0 min (0 m). Values are means \pm SEM ($*P < 0.05$, $n = 3$). Primary RGCs were isolated from 12-day-old pups; primary astrocytes were isolated from 3-day-old pups. (B) Western blot analysis of extracts from primary RGCs and astrocytes (25 μ g), treated with TNF (30 ng/mL) or PBS (control) in a time-dependent manner, using I κ B α . TNF-treated astrocytes demonstrated I κ B α degradation peaking at 15–30 min, while I κ B α was not degraded in RGC lysates. GAPDH was used as a loading control. (C and D) Quantification of the data shown in B: (i) the I κ B α band intensities were normalised to GAPDH, and then (ii) the results were expressed as percentages of the corresponding value in the cells treated for 0 min (0 m). Values are means \pm SEM ($n = 3$, $**P < 0.01$).

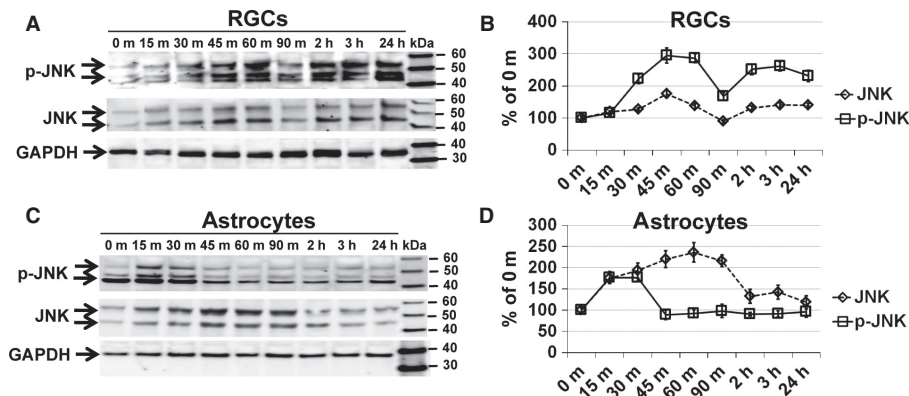


FIG. 4. Representative (A and C) Western blots and (B and D) densitometric analysis of JNK (total) and phosphorylated JNK (p-JNK) protein levels in the TNF-treated primary RGCs and astrocytes revealed sustained JNK activation in TNF-treated RGCs, while primary astrocytes demonstrated transient JNK activation. Whole-cell extracts (25 μ g) were collected from zero to 90 min as well as 2, 3 and 24 h after TNF treatment, and the JNK state was tested by Western blot analysis using an antibody against JNK and p-JNK. The JNK and p-JNK intensities were normalised to GAPDH and the results expressed as percentages of the corresponding values in the cells treated for 0 min (0 m). Values are means \pm SEM; $n = 3$.

parisons. In the case of single comparisons, Student’s *t*-test was applied. The data are presented as mean \pm SEM. *P*-values ≤ 0.05 were considered statistically significant.

Results

In our study we used primary RGCs isolated according to the two-step immunopanning protocol and primary astrocytes obtained by the ‘shaking method’. Isolated primary RGCs and astrocytes were plated on cover slips in a 24-well plate and were treated with 30 ng/mL of TNF. Cultures of primary RGCs were assessed for levels of necrotic, apoptotic and living cells after 24 h. Quantification of

RGC survival showed significantly higher levels of cell survival in cultures treated with PBS (control), rather than with TNF ($68 \pm 1\%$ vs. $55 \pm 1\%$, $t_4 = 8.8$, $P = 0.0001$; Fig. 1). Importantly, RGC cultures treated with TNF chiefly underwent necrotic cell death ($34 \pm 1\%$ of TNF-treated cells compared to $8 \pm 2\%$ of PBS-treated cells; $t_4 = 12.4$, $P = 0.00002$; Fig. 1A). When astrocyte loss was evaluated in cultures treated with TNF and PBS (control), there was no significant astrocyte death detected in TNF- or PBS-treated cultures (Fig. 1).

To explain why TNF treatment promoted RGC death but had no effect on astrocytes, we noted that NF- κ B transcriptional factor promotes cell survival by mediating production of anti-apoptotic factors

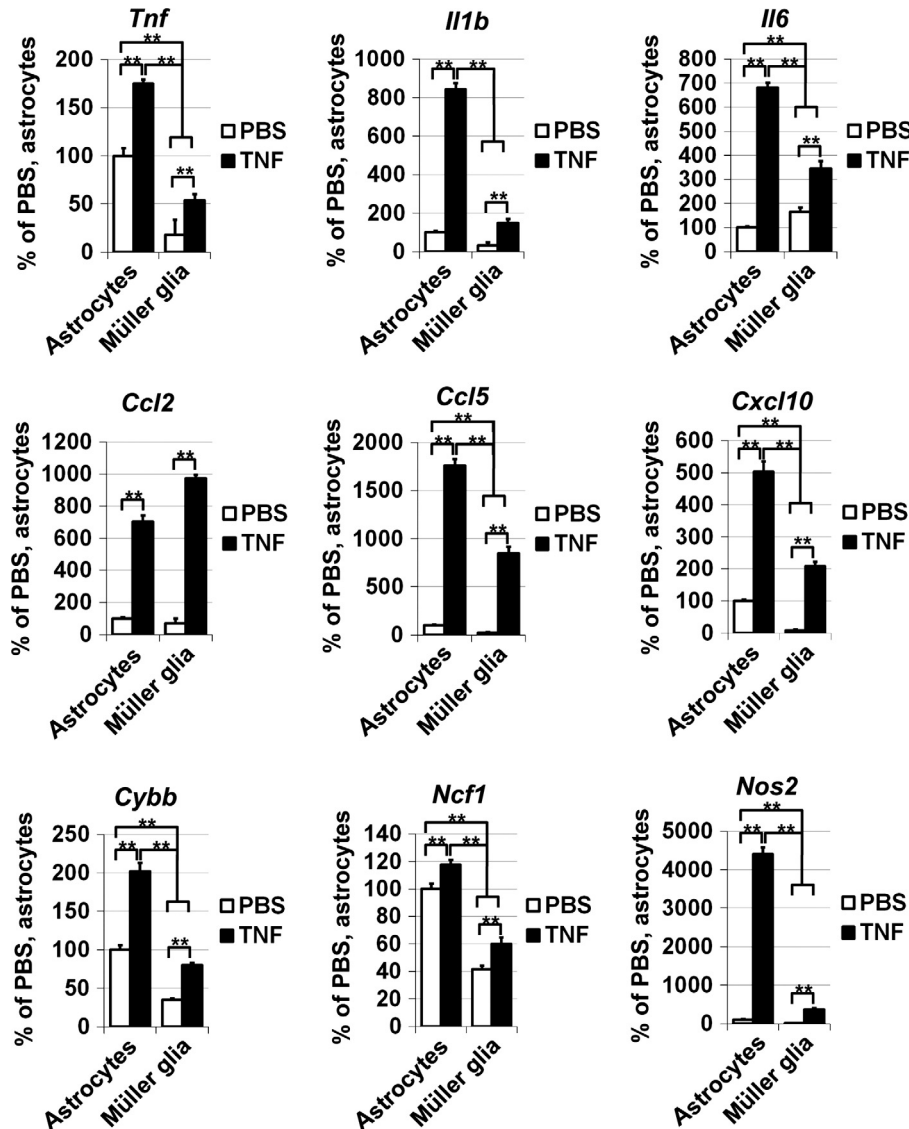


FIG. 5. Stimulation of primary astrocytes with 30 ng/mL of TNF led to significantly higher expression of pro-inflammatory genes than in TNF-treated Müller glia. Gene expression was assessed in primary astrocytes and Müller glia treated with TNF and PBS (controls) for 24 h by quantitative RT-PCR (six biological repeats). For each gene, results are expressed as percentages \pm SEM of the corresponding values in the astrocytes treated with PBS ($n = 6$, $**P < 0.01$).

(Mattson & Meffert, 2006). Therefore, we asked whether the activity of NF- κ B transcriptional factor is present in astrocytes but not in RGCs. First, we evaluated the expression of members of the NF- κ B family in isolated RGCs and astrocytes. We found that transcripts of all members of the NF- κ B family were present in RGCs and astrocytes (Fig. 2A). Because NF- κ B transcriptional factor, as a heterodimer comprising p65 (Rela) and p50 (Nfkb1) subunits, promotes cell survival, we tested for the presence of these proteins in RGCs and astrocytes treated with TNF (30 ng/mL). The cell lysates were collected in a time-dependent manner and proteins were detected by Western blotting. Our data indicated that both subunits were present at each tested time point (Fig. 2B). Next, we measured the levels of NF- κ B activity in nuclear extracts of TNF-treated primary RGC and astrocyte cultures using the NF- κ B p65 transcription factor ELISA kit. The time-course analysis revealed sustained NF- κ B activation in glial cells, while NF- κ B activity in TNF-treated RGCs was not detected (Fig. 3A). As I κ B degradation is required for the translocation of active NF- κ B from the cytoplasm to the nucleus (Mattson &

Meffert, 2006), we evaluated the levels of I κ B subunit α (I κ B α) in TNF-treated RGC and astrocyte lysates in a time-dependent manner by Western blot analysis. No I κ B α degradation was detected, but we did observe an increase in I κ B α level in RGC cultures after 30 min of TNF treatment (Fig. 3B). In astrocytes, I κ B α levels were significantly reduced after 15 and 30 min of TNF treatment (Fig. 3B). The levels of I κ B α in these cultures were restored later (Fig. 3B). It should be noted that these results correlated with the measured NF- κ B activity (Fig. 3A). Thus, our findings suggest that TNF-treated RGCs have an impaired ability to activate NF- κ B that was associated with TNF-mediated RGC death. Primary astrocyte cultures exhibit NF- κ B activation after TNF treatment, accompanied by astrocyte survival.

It has been shown previously that sustained JNK activation (phosphorylation) facilitates cell death when NF- κ B activation is blocked (Kamata *et al.*, 2005; Papa *et al.*, 2006). As RGCs failed to activate NF- κ B after TNF treatment, we asked whether TNF mediates sustained activation of JNK in RGCs but not in astrocytes. To answer

this question, first we treated primary RGC and astrocyte cultures with TNF in a time-dependent manner. Next, levels of JNK phosphorylation (activation) were evaluated in cell lysates by Western blot analysis. We also tested total JNK levels as well as GAPDH levels as loading controls. The time-course analysis revealed sustained JNK phosphorylation in RGCs (Fig. 4A and B). Meanwhile, TNF-treated astrocytes demonstrated transient JNK activation peaking at 15–30 min (Fig. 4C and D). It should be noted that while total levels of JNK were more increased between 15 and 90 min compared to 0 min and 24 h, the levels of phosphorylated JNK were more reduced between 45 min and 24 h than at the 15- to 30-min time interval (Fig. 4C and D). Collectively, our data indicate that JNK signaling is activated in opposite ways in neurons and astrocytes in the presence of TNF. While TNF-treated astrocytes just demonstrated transient JNK phosphorylation (activation), TNF promoted sustained activation of JNK in RGCs that was associated with increased neuronal death.

Our last step was to test the expression of pro-inflammatory factors in TNF-treated astrocytes, because astroglial NF- κ B controls many genes involved in inflammation. As Müller glia represent the most abundant type of glial cells in the retina, we also tested expression of pro-inflammatory genes in TNF-treated primary Müller glial cells (Bringmann *et al.*, 2006). To this end, primary astrocytes and Müller cells were treated with 30 ng/mL of TNF over 24 h. Cells were collected after treatment and expression of genes coding for cytokines, chemokines and enzymes producing reactive oxygen and nitrogen species was analysed by quantitative RT-PCR. We observed a higher increase in the levels of expression of *Tnf* ($t_6 = 12.2$, $P = 2 \times 10^{-6}$), *Il1b* ($t_6 = 22.2$, $P = 2 \times 10^{-10}$), *Il6* ($t_6 = 28.2$, $P = 4 \times 10^{-10}$) cytokines, *Ccl2* ($t_6 = 15.2$, $P = 3 \times 10^{-8}$), *Ccl5* ($t_6 = 24.2$, $P = 2 \times 10^{-9}$), *Cxcl10* ($t_6 = 12.5$, $P = 2 \times 10^{-7}$) chemokines, inducible nitric oxide synthase (*Nos2*; $t_6 = 24.2$, $P = 3 \times 10^{-10}$), and of subunits of NAD(P)H oxidase [*Cybb* ($t_6 = 8.07$, $P = 1 \times 10^{-5}$) and *Ncf1* ($t_6 = 3.28$, $P = 0.0083$)] in TNF-treated astrocytes than in the PBS-treated control (Fig. 5). We also detected increased expression of *Tnf* ($t_6 = 5.07$, $P = 6 \times 10^{-4}$), *Il1b* ($t_6 = 4.61$, $P = 9 \times 10^{-4}$), *Il6* ($t_6 = 4.94$, $P = 8 \times 10^{-4}$), *Ccl2* ($t_6 = 22.2$, $P = 4 \times 10^{-9}$), *Ccl5* ($t_6 = 12.1$,

$P = 3 \times 10^{-7}$), *Cxcl10* ($t_6 = 13.7$, $P = 9 \times 10^{-8}$), *Nos2* ($t_6 = 8.89$, $P = 9 \times 10^{-6}$), *Cybb* ($t_6 = 13.5$, $P = 1 \times 10^{-7}$) and *Ncf1* ($t_6 = 3.31$, $P = 0.0079$) in TNF-treated Müller glia compared to PBS-treated controls (Fig. 5). However, the expression of neurotoxic cytokines, chemokines and genes coding reactive oxygen and nitrogen species producing enzymes was significantly reduced in TNF-treated Müller glia relative to TNF-treated astrocytes (Fig. 5). Thus, our data suggest that TNF promotes the neurotoxic astroglial pro-inflammatory response, while contribution of Müller glia in TNF-mediated neurotoxicity is insignificant.

Discussion

TNF is involved in the CNS immune response to any neural damage, and therefore plays an important role in many neurodegenerative conditions, especially those in the retina (De Simoni & Imeri, 1998; Perry *et al.*, 2002; Berger *et al.*, 2008; McCoy & Tansey, 2008; Tezel, 2008). TNF and TNF-regulated signaling cascades play a deleterious role in glaucoma and retinal ischemia, facilitating RGC death (Berger *et al.*, 2008; Tezel, 2008; Dvorianchikova *et al.*, 2009; Barakat *et al.*, 2012). Although cell culture data indicate that TNF and its signaling cascades can mediate neurotoxicity, the data also indicates their protective functions (Kamata *et al.*, 2005; Marques-Fernandez *et al.*, 2013). To explain these controversial results, we performed this study. We investigated the activation of TNF-dependent signaling cascades in RGCs and in astrocytes, which play a critical role in neuronal survival and death under normal and pathological conditions (Varela & Hernandez, 1997; Fields & Stevens-Graham, 2002; Pekny & Nilsson, 2005; Dvorianchikova *et al.*, 2009; Son *et al.*, 2010; Barakat *et al.*, 2012). We found that TNF activated NF- κ B in astrocytes but it did not activate NF- κ B in RGCs. We also found that TNF mediated sustained activation of JNK in RGCs, while it initiated transient activation of JNK in astrocytes. These changes in activation of the signaling cascades were associated with an increased level of neuronal death, while glial cells demonstrated significant survival after TNF treatment. We also found that TNF-mediated NF- κ B activation in astrocytes was accompanied by an increased expression of pro-inflammatory factors by these cells.

There is already no doubt that NF- κ B activation and translocation from the cytoplasm to the nucleus is a necessary and probably sufficient condition for the survival of the cell subjected to stress, as NF- κ B mediates the production of a number of cellular inhibitors of stress-mediated apoptosis (Mattson & Meffert, 2006). In addition, NF- κ B activity helps the cell to avoid the sustained phase of JNK activation and, thus, promotes cell survival (Kamata *et al.*, 2005; Nakano *et al.*, 2006; Papa *et al.*, 2006; Weston & Davis, 2007). NF- κ B can also induce the expression of a number of genes in astrocytes, whose products can mediate pro-inflammatory toxicity in the retina (Dvorianchikova *et al.*, 2009; Barakat *et al.*, 2012). Pro-survival activity of NF- κ B in astrocytes is most probably necessary for avoiding self-generated toxicity and for survival in these conditions. Our data indicate that, in the presence of TNF, astrocytes initiated the activation of NF- κ B, which was associated with astrocyte survival and increased expression of pro-inflammatory factors (including TNF). At the same time, our data indicate that absence of I κ B degradation in RGCs prevents NF- κ B activation after TNF treatment, and this phenomenon was associated with sustained JNK activation and RGC death, predominantly by necrosis. Previously, necrosis was viewed as an accidental and unregulated cellular event. However, we now know that necrosis, like apoptosis, can be executed by programmed mechanisms (Vanden Berghe *et al.*, 2014).

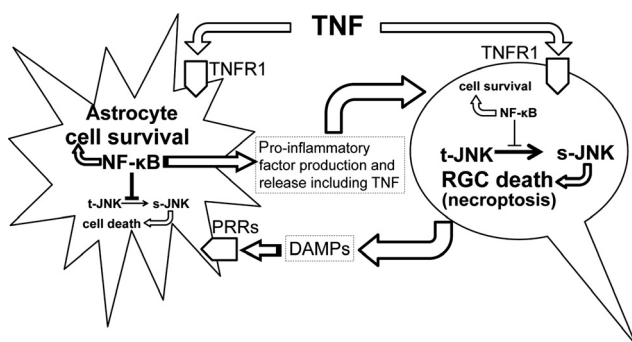


Fig. 6. Proposed mechanism of TNF-induced neuronal toxicity in the retina: TNF mediates NF- κ B activation in astrocytes, which facilitates astroglial survival but also initiates production of neurotoxic pro-inflammatory factors such as TNF in the retina. The lack of NF- κ B activity in RGCs in the presence of TNF, as well as sustained JNK activation, both contribute to RGC death, mostly by necrosis (necroptosis). Meanwhile, endogenous factors (called damage-associated molecular patterns or DAMPs) liberated from necrotic RGCs can activate astrocyte pattern recognition receptors (PRRs) and mediate production of neurotoxic pro-inflammatory factors such as TNF. Thus, TNF-mediated RGC necrosis can increase the neurotoxic astrocyte response and can therefore indirectly promote RGC death. t-JNK, transient JNK activation; s-JNK, sustained JNK activation.

This form of necrotic cell death is called necroptosis. The role of the TNF signaling cascade was the first to be shown in programmed necrosis (necroptosis) (Vanden Berghe *et al.*, 2014). TNF can mediate programmed necrosis in cells that failed to activate the pro-survival NF- κ B signaling cascade and apoptosis (Vanden Berghe *et al.*, 2014). It should also be noted that sustained JNK activation contributes to necrotic cell death (Kamata *et al.*, 2005; Nakano *et al.*, 2006; Papa *et al.*, 2006; Kim *et al.*, 2007; Weston & Davis, 2007; Morgan *et al.*, 2008). Importantly, we previously demonstrated that RGCs underwent necroptosis after ischemia–reperfusion (IR) in the retina (Dvorianchikova *et al.*, 2014a). As TNF is released and mediates retinal damage after IR (Berger *et al.*, 2008), TNF may activate signaling cascades that facilitate RGC programmed necrosis (necroptosis). Therefore, sustained JNK activation and the inability of RGCs to activate NF- κ B should both facilitate RGC necroptosis in the presence of TNF.

As Müller glia are the most abundant type of glial cell in the retina and can contact all retinal neurons (including RGCs; Bringmann *et al.*, 2006), we also tested for a pro-inflammatory response of Müller glia mediated by TNF. We detected that the level of expression of neurotoxic pro-inflammatory factors in TNF-treated Müller glia was significantly lower than in TNF-treated astrocytes. However, can such a response of Müller glia harm the retina? If Müller glia-related toxicity could mediate neuronal death then all retinal neurons should be affected and die as a result of Müller glia activity, because these cells can contact all retinal neurons (Bringmann *et al.*, 2006). Thus, the neurotoxic activity of Müller glia has to mediate damage in all retinal layers. However, while almost every retinal disease is associated with a reactive Müller cell gliosis, it has never been shown to affect all retinal layers (Bringmann *et al.*, 2006). In our previous studies, when we inactivated NF κ B signaling in GFAP-expressing retinal cells (astrocytes and Müller glia), we found significant survival of RGCs (Dvorianchikova *et al.*, 2009; Barakat *et al.*, 2012; Brambilla *et al.*, 2012). Thus, this can be a result of inactivation of NF κ B in GFAP-positive cells, which only belong to the ganglion cell and nerve fiber layers of the retina. Astrocytes are GFAP-positive cells and reside only among RGCs and their axons in the ganglion cell and nerve fiber layers of the retina, and make direct contact with RGCs. Therefore, only the cytotoxic activity of astrocytes can explain the observed data (Dvorianchikova *et al.*, 2009; Barakat *et al.*, 2012; Brambilla *et al.*, 2012). Thus, TNF-mediated astroglial toxicity should play a very significant role in RGC death, as compared to TNF-mediated Müller glia toxicity. This was the reason we chose astrocytes over Müller glia in our study.

As retinal tissue contains RGCs and astrocytes in close proximity, we can propose a model of TNF toxicity in the retina (Fig. 6). TNF produced in the retina as a result of neurodegenerative conditions promotes sustained JNK activation in RGCs which, in the absence of NF- κ B activity, mediates RGC death, chiefly by necrosis. While NF- κ B activation in astrocytes promotes survival of these cells, it initiates a toxic pro-inflammatory response in the retina. This response creates even worse conditions in the retina and increases RGC death. In addition, endogenous factors liberated from necrotic RGCs can be recognised by astrocytes as signals for ‘danger’ (so called damage-associated molecular patterns or DAMPs; Challa & Chan, 2010; Piccinini & Midwood, 2010; Dvorianchikova *et al.*, 2011, 2014b). After engaging the DAMPs, pattern recognition receptors such as toll-like receptors (e.g. TLR4) activate signaling cascades, which trigger inflammation and damage (Challa & Chan, 2010; Piccinini & Midwood, 2010; Dvorianchikova *et al.*, 2011, 2014b). In other words, if the cell dies by necrosis, this event leads

to pro-inflammatory toxicity that stimulates death of the surrounding cells that have survived the initial stress. As such, TNF-mediated RGC necrosis can increase the neurotoxic astrocyte response, thereby indirectly promoting RGC death. This model can explain the observed contradictions. In particular, this model and our findings can explain the reason that inhibition of astroglial NF- κ B protects against damage and neuronal loss in experimental models of retinal neurodegenerative diseases (Dvorianchikova *et al.*, 2009; Barakat *et al.*, 2012; Brambilla *et al.*, 2012).

In conclusion, available evidence appears to indicate that the effects of TNF as well as NF- κ B and JNK signaling cascades activated by TNF in the retina are cell-specific and, as a result, more complicated than previously believed to be. Our findings suggest that in the presence of TNF, NF- κ B and JNK signaling cascades are activated in opposite ways in RGCs and astrocytes, which contribute to RGC death. Thus, the design of effective therapy for patients suffering from retinal neurodegenerative diseases should consider the cell type-specific beneficial and detrimental effects of TNF signaling.

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Abbreviations

CNS, central nervous system; I κ B α , I κ B subunit α ; JNK, c-Jun N-terminal kinase; NF- κ B, nuclear factor kappa B; RGC, retinal ganglion cell; RT-PCR, reverse transcription–polymerase chain reaction; TNF, tumor necrosis factor-alpha.

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