

Assessment of Neurotrophins and Inflammatory Mediators in Vitreous of Patients With Diabetic Retinopathy

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Submitted: March 30, 2017

Accepted: September 18, 2017

Citation: Boss JD, Singh PK, Pandya HK, et al. Assessment of neurotrophins and inflammatory mediators in vitreous of patients with diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2017;58:5594–5603. DOI: 10.1167/iovs.17-21973

PURPOSE. To assess vitreous levels of inflammatory cytokines and neurotrophins (NTs) in diabetic retinopathy (DR) and elucidate their potential roles.

METHODS. A prospective study was performed on 50 vitreous samples obtained from patients with DR ($n = 22$) and the nondiabetic controls ($n = 28$). All patients were candidates for vitrectomy. Inflammatory cytokine and NT levels were determined with ELISA. Potential source and role of NTs was determined by using human retinal Müller glia and mouse photoreceptor cells and challenging them with TNF- α or IL-1 β , followed by detection of NTs and cell death.

RESULTS. Vitreous NT levels of all DR patients were significantly higher than those of nondiabetic controls (nerve growth factor [NGF; $P = 0.0001$], brain-derived neurotrophic factor [BDNF; $P = 0.009$], neurotrophin-3 [NT-3; $P < 0.0001$], neurotrophin-4 [NT-4; $P = 0.0001$], ciliary neurotrophic factor [CNTF; $P = 0.0001$], and glial cell-derived neurotrophic factor [GDNF; $P = 0.008$]). Similarly, the levels of inflammatory mediators IL-1 β ($P < 0.0001$), IL-6 ($P = 0.0005$), IL-8 ($P < 0.0001$), and TNF- α ($P < 0.0001$) were also higher in eyes with DR. Interestingly, inflammatory cytokine and NT levels, particularly TNF- α ($P < 0.05$), IL-8 ($P < 0.004$), NT-3 ($P = 0.012$), NGF ($P = 0.04$), GDNF ($P = 0.005$), and CNTF ($P = 0.002$), were higher in eyes with nonproliferative diabetic retinopathy (NPDR) than in eyes with active proliferative diabetic retinopathy (PDR). Cytokine stimulation of Müller glia resulted in production of NTs, and GDNF treatment reduced photoreceptor cell death in response to inflammation and oxidative stress.

CONCLUSIONS. Together, our study demonstrated that patients with DR have higher levels of both inflammatory cytokines and NTs in their vitreous. Müller glia could be the potential source of NTs under inflammatory conditions to exert neuroprotection.

Keywords: diabetic retinopathy, inflammation, neurotrophins, vitreous humor, human

Diabetic retinopathy (DR) is a disease of progressive vascular neurodegeneration and it remains a significant cause of blindness in middle-aged adults in the United States.¹ Although the pathogenesis of DR is likely multifactorial and remains unknown, it appears to be associated with increased inflammation induced by oxidative stress,² generation of reactive oxygen species,³ advanced glycation end products, and a plethora of inflammatory mediators.⁴ Several clinical studies have demonstrated that diabetics have increased serum levels of inflammatory markers, including C-reactive protein, interleukin 6 (IL-6), and tumor necrosis factor- α (TNF- α), suggesting that inflammatory processes play a considerable role in the pathogenesis of DR.⁵ Inflammation is generally considered a beneficial host response toward invading pathogens or tissue injury.⁶ Prolonged inflammation, as in diabetes, however, can be destructive and maladaptive, leading to irreversible damage to delicate tissues, such as the retina.⁷ Indeed, DR exhibits features of chronic neuroinflammation, but the precise relationship between inflammatory alterations in DR and the loss of neural function is currently being investigated.^{8,9}

More recently, in addition to being a vascular complication of diabetes, DR has been recognized as a neurodegenerative disease with reported vision deficits at the onset of diabetes.¹⁰ The neuronal changes in DR include retinal cell death, loss of ganglion cells,¹¹ reduced inner retinal thickness, neurofilament abnormality, and declined ERG.^{10,11} These physiological conditions trigger neuronal survival signaling, including the production of neurotrophins (NTs).¹² Given the critical role played by NTs in regulating neuronal functions, it is not surprising then that neurodegenerative disorders, including DR, are associated with altered NT levels and/or expression pattern of their receptors.

NT levels have been shown to be elevated in vitreous samples in animal studies with induced proliferative diabetic retinopathy (PDR).¹³ While glial cell line-derived neurotrophic factor (GDNF)¹⁴ has been shown to be increased in vitreous samples of patients with DR, to our knowledge, a comprehensive analysis of other NT levels, including levels of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and



ciliary neurotrophic factor (CNTF), have not been evaluated in DR. Therefore, the objectives of our study were (1) to analyze and compare NT levels in the vitreous of patients with or without DR, (2) to find correlation between the levels of vitreous NTs and inflammatory mediators in patients with DR, (3) to analyze the effect of panretinal photocoagulation (PRP) ablation of the ischemic peripheral retina in DR on NT levels, and (4) to determine the potential mechanism of NT production and its functional role under inflammatory/oxidative stress conditions.

MATERIALS AND METHODS

Patient Population and Vitreous Sample Collection

This was a prospective study conducted according to the tenets of the Declaration of Helsinki. All patients were candidates for vitrectomy and had signed a preoperative informed consent with their approval to use the excised vitreous fluid for analysis and clinical research. The study design and protocol were approved by the Wayne State University School of Medicine Institutional Review Board. Exclusion criteria included patients younger than 18 years, a history of previous intravitreal anti-vascular endothelial growth factor (VEGF) injections, a history of or current uveitis, current or recent topical or systemic steroid use, a history of penetrating eye injury, a history of blunt trauma to the eye, or previous intraocular surgery within the past 6 months. Patients were excluded from analysis of intravitreal inflammatory cytokines if they underwent a pars plana lensectomy for retained lens fragments due to complicated cataract surgery.

Undiluted vitreous fluid samples were obtained from 50 eyes from 50 patients (DR, $N=22$; Nondiabetic, $N=28$) from 2013 to 2015 during pars plana vitrectomy. The indications for vitrectomy were rhegmatogenous and tractional retinal detachment, nonclearing vitreous hemorrhage, epiretinal membrane peel, macular hole repair, or retained lens fragments. (Table 1) The preoperative degree of DR was graded from the current preferred practice patterns outlined by the American Academy of Ophthalmology. Undiluted vitreous samples were collected by manual suction into a syringe through the aspiration line of vitrectomy before opening the infusion line. Samples were transferred to sterile polypropylene screw cap conical bottom vials and were immediately snap-frozen in liquid nitrogen until transfer to a -80°C freezer for storage and assays.

Vitreous Neurotrophins and Cytokine Analysis

Vitreous levels of NGF (Cat. No. DY 256), BDNF (Cat. No. DY248), NT-3 (Cat. No. DY267), NT-4 (Cat. No. DY268), CNTF (Cat. No. DY257), GDNF (Cat. No. DY212), interleukin 1 β (IL-1 β , Cat. No. DY201), IL-6 (Cat. No. DY206), IL-8 (Cat. No. DY208), and TNF- α (Cat. No. DY210) were determined by ELISA as per manufacturer's instruction (R&D Systems, Minneapolis, MN, USA). For ELISA assay, total protein concentration in the vitreous was determined by the Micro BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) and equal amount (20 μg) of protein was used for ELISA assay.

Cell Culture and Treatment

The immortalized human Müller glial cells (MIO-M1 cell line)¹⁵ were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin, and

TABLE 1. Surgical Indications for Vitrectomy

Surgical Indication	NPDR, No. of Eyes	PDR, No. of Eyes	Control, No. of Eyes
TRD	0	5	0
RRD	4	0	9
ERM	3	2	4
NCVH	2	8	2
MH	1	0	6
RLF	0	0	7

Five eyes with PDR met surgical criteria for having both an NCVH and TRD. ERM, epiretinal membrane; MH, macular hole; NCVH, nonclearing vitreous hemorrhage; RLF, retained lens fragment; RRD, rhegmatogenous retinal detachment; TRD, tractional retinal detachment.

L-glutamine (10 $\mu\text{g}/\text{mL}$). Cells were grown in serum-free media before TNF- α challenge. For NT expression, MIO-M1 cells were challenged with recombinant TNF- α (100 ng/mL), IL-1 β (100 ng/mL), IL-4 (100 ng/mL), or dexamethasone (100 μM) for indicated time points. The conditioned media was centrifuged at 10,000g for 10 minutes to remove cell debris. The clear supernatant was used for dot blot to measure the expression of various NTs.

For the neuroprotection study, mouse cone photoreceptor cell line 661W, as described in our previous study,¹⁶ was used. The 661W cell line was maintained in DMEM supplemented with 10% FBS, 10 $\mu\text{g}/\text{mL}$ L-glutamine, 1% penicillin-streptomycin, 40 $\mu\text{g}/\text{L}$ hydrocortisone, 40 $\mu\text{g}/\text{L}$ progesterone, 32 mg/L putrescine, and 40 $\mu\text{L}/\text{L}$ β -mercaptoethanol. Photoreceptor cell death was induced by challenging 661W cells with H₂O₂ (100 μM) or TNF- α (100 ng/mL) for 24 hours in the presence and absence of GDNF (100 ng/mL). The cell death and protection by GDNF treatment was assessed by TUNEL staining using an ApopTag fluorescein in situ apoptosis detection kit as described previously.¹⁷

Dot Blot Analysis

To measure the NT level in MIO-M1, dot blot was performed as described previously.¹⁵ Briefly, 50 μL conditioned media was loaded onto a 0.2- μm nitrocellulose membrane by using a BIO-DOT apparatus (Bio-Rad, Hercules, CA, USA) and vacuum suction. The membrane was fixed in 10% formaldehyde in Tris buffer saline (TBS) for 1 hour at room temperature (RT). The membrane was blocked in 5% skim milk made in TBST (TBS containing 0.05% Tween 20) for 1 hour at RT and incubated with primary antibody for various NTs (GDNF, NGE, NT-3, and NT-4) overnight at 4 $^{\circ}\text{C}$. On the following day, the blot was washed three times in TBST and incubated with respective anti-mouse or anti-rabbit horseradish peroxidase (HRP) conjugates (Bio-Rad) for 1 hour at RT. The blot was developed using SuperSignal West Femto maximum Sensitivity Substrate (Thermo Scientific) via chemiluminescence using a Kodak image station, 4000R Pro molecular imaging system (Carestream Health, Inc., Rochester, NY, USA). Dot intensity was quantified using ImageJ analysis software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Values of vitreous concentrations of cytokines and NTs were reported as mean \pm standard deviation (SD). All of the analyses were performed by using the Statistical Package for the Social

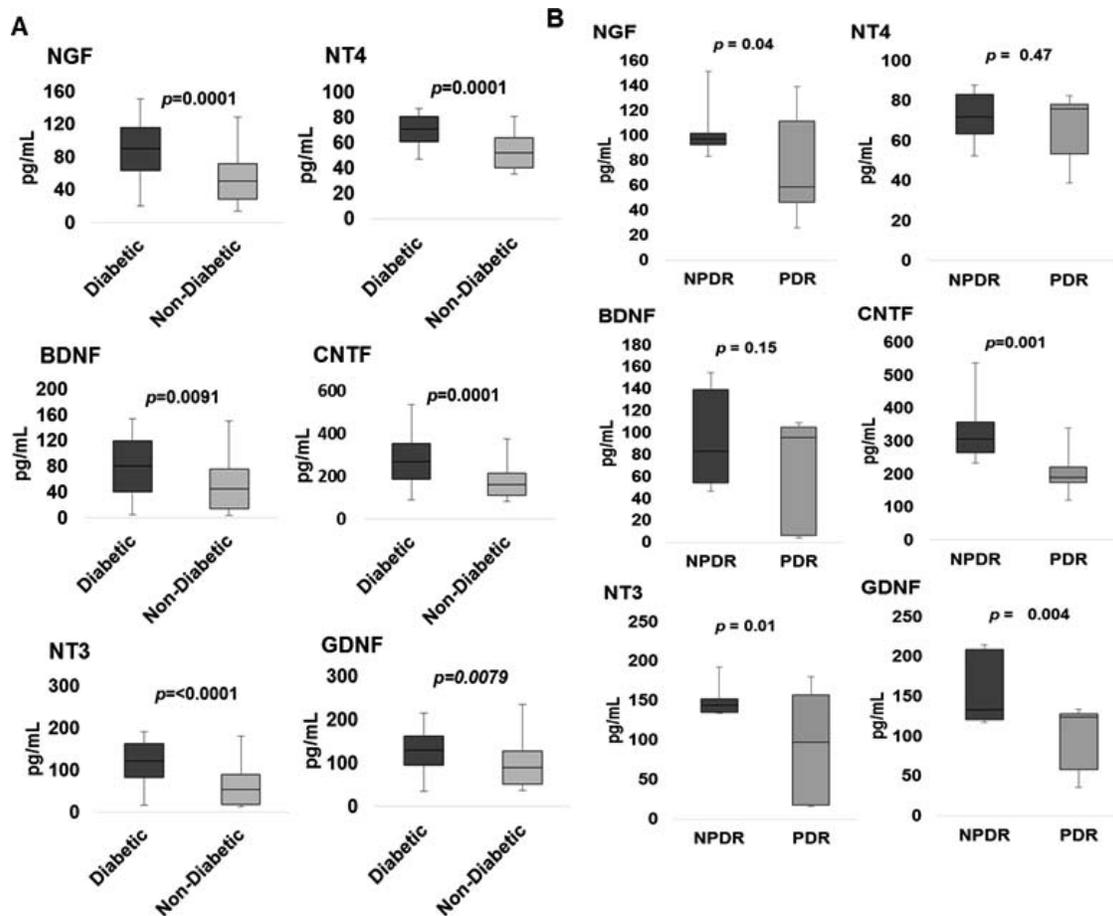


FIGURE 1. Box-and-whisker plot analysis showing comparison of vitreous NGF, BDNF, NT-3, NT-4, CNTF, and GDNF levels in diabetics ($n = 22$) compared to nondiabetics ($n = 28$) (A) and diabetic patients with NPDR compared to active PDR (B).

Science software version 16.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad prism V7.02 (GraphPad Software, La Jolla, CA, USA). The unpaired t -test was used for the normally distributed continuous variables, comparing the means from two independent groups, and a P value < 0.05 was considered statistically significant. One-way ANOVA was performed for the dot blot densitometry analysis. The relationship between NTs and cytokines was determined by Spearman rank correlation.

RESULTS

Demographics

A total of 50 vitreous samples from 50 patients (50 eyes) were collected. Among these 22 patients had DR and 28 patients were nondiabetic controls. The mean age of all subjects was 64.7 years (range, 42–90) and 39 patients were male and 11 were female. Among diabetics, the mean age was 67.8 years (range, 46–90), whereas the mean age of nondiabetics was 61.5 years (range, 42–78). Among patients with DR, at the time of vitrectomy 3 had mild NPDR, 4 had moderate NPDR, 3 had severe NPDR, and 12 had PDR (Table 1). All patients were candidates for vitrectomy performed at the Kresge Eye Institute of Wayne State University, Detroit, Michigan, United States.

Vitreous Levels of Neurotrophins and Inflammatory Cytokines

Our analysis showed that NTs, including NGF, BDNF, NT-3, NT-4, CNTF, and GDNF, were present in all vitreous samples of diabetic and nondiabetic eyes, but levels were on average higher in DR eyes than in nondiabetic eyes. There was a significant increase in levels of NGF ($P = 0.0001$), BDNF ($P = 0.0091$), NT-3 ($P < 0.0001$), NT-4 ($P = 0.0001$), CNTF ($P = 0.0001$), and GDNF ($P = 0.0079$) in eyes with DR compared to nondiabetic eyes (Fig. 1A; Table 2). Among eyes with DR, eyes with NPDR compared to eyes with active PDR had higher vitreous concentrations of NGF ($P = 0.04$), BDNF ($P = 0.15$), NT-3 ($P = 0.01$), NT-4 ($P = 0.48$), CNTF ($P = 0.001$), and GDNF ($P = 0.004$) (Fig. 1B). NTs in eyes with NPDR compared to nondiabetic eyes had higher average vitreous concentrations of NGF ($P < 0.0001$), BDNF ($P < 0.0001$), NT-3 ($P < 0.0001$), NT-4 ($P < 0.0001$), CNTF ($P < 0.0001$), and GDNF ($P < 0.0001$). The Spearman correlation analysis revealed a strong correlation (0.513; $P < 0.0001$) between NT and cytokine levels in patients with DR. The subgroup analysis also revealed a strong correlation between NTs and cytokines in NPDR (0.366; $P = 0.02$) and PDR (0.535; $P = 0.001$).

The assessment of inflammatory cytokines revealed overall higher levels in eyes with DR than in nondiabetic controls (Fig. 2). There was a significant increase in levels of IL-1 β ($P < 0.0001$), IL-6 ($P = 0.0005$), IL-8 ($P < 0.0001$), and TNF- α ($P < 0.0001$) in eyes with DR compared to nondiabetic eyes (Fig. 2A). The comparative analysis of cytokine levels in eyes with

TABLE 2. Quantitative Analysis and Comparison of Intravitreal Levels of Various Neurotrophins in Eyes With Diabetic Retinopathy ($N = 22$) and Nondiabetic Eyes ($N = 28$)

Neurotrophin	Diabetic, Mean \pm SD, pg/mL	Nondiabetic, Mean \pm SD, pg/mL	P Value
NGF	90.6 \pm 35.1	51.0 \pm 31.2	0.0001
BDNF	80.2 \pm 48.3	45.5 \pm 41.8	0.009
NT-3	122.9 \pm 57.0	53.9 \pm 49.1	<0.0001
NT-4	71.4 \pm 12.3	52.7 \pm 13.6	0.0001
CNTF	270.4 \pm 110.0	161.8 \pm 71.8	0.0001
GDNF	129.0 \pm 50.8	89.5 \pm 49.4	0.008
IL-1 β	12.9 \pm 5.8	4.0 \pm 1.8	<0.0001
IL-6	212.5 \pm 179.6	43.3 \pm 84.7	0.0005
IL-8	53.6 \pm 28.1	18.2 \pm 9.8	<0.0001
TNF- α	155.8 \pm 82.0	63.9 \pm 23.8	<0.0001

SD, standard deviation; pg/mL, picograms/milliliter; NGF, nerve growth factor; BDNF, brain derived neurotrophic factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; CNTF, ciliary neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; IL-1 β , Interleukin 1 beta; IL-6, Interleukin 6; IL-8, Interleukin 8; TNF α , tumor necrosis factor alpha.

NPDR ($N = 12$, excluding eyes with PDR) and in nondiabetic control eyes ($N = 21$, excluding retained lens fragment eyes) also revealed a significant increase in levels of IL-1 β ($P < 0.0001$), IL-6 ($P = 0.0011$), IL-8 ($P < 0.0001$), and TNF- α ($P < 0.001$). Interestingly, levels of some cytokines were higher in

diabetic eyes with NPDR than with active PDR, with an increase in levels of IL-8 ($P = 0.004$) and TNF- α ($P < 0.05$), while IL-1 β ($P = 0.08$) and IL-6 ($P = 0.41$) concentrations were comparable (Fig. 2B).

In a subgroup analysis of eyes with severe NPDR and PDR, nine (60%) vitreous samples were collected from eyes with a history of PRP, and six (40%) control samples were collected from eyes without a history of PRP (No PRP). The average patient age was 57.9 years (range, 44–76) in the PRP group and 65.3 years (range, 42–71) in No PRP. The comparative analysis of PRP versus No PRP revealed no significant difference in the vitreous levels of either NTs (Fig. 3A; Table 3) or inflammatory cytokines (Fig. 3B).

Müller Glia Secrete Neurotrophins in Response to Inflammatory Stimuli

We previously reported that in response to infectious or inflammatory stimuli, retinal glial cells (Müller glia and microglia) get activated and produce more inflammatory mediators along with protective molecules.^{15,18,19} We hypothesized that diabetes-induced increase in inflammatory mediators triggers the production of NTs by Müller glia, the major glial cell types in the retina.²⁰ To test this, we challenged human retinal Müller glia (MIO-M1 cell line) with recombinant TNF- α or IL-1 β for 8 and 24 hours. Our data showed that both TNF- α and IL-1 β -challenged Müller glia secrete NTs in conditioned media (Figs. 4A, 4B). To determine whether TNF- α or IL-1 β -stimulated NT production is specific to inflammatory stimuli, MIO-M1 cells were challenged with an

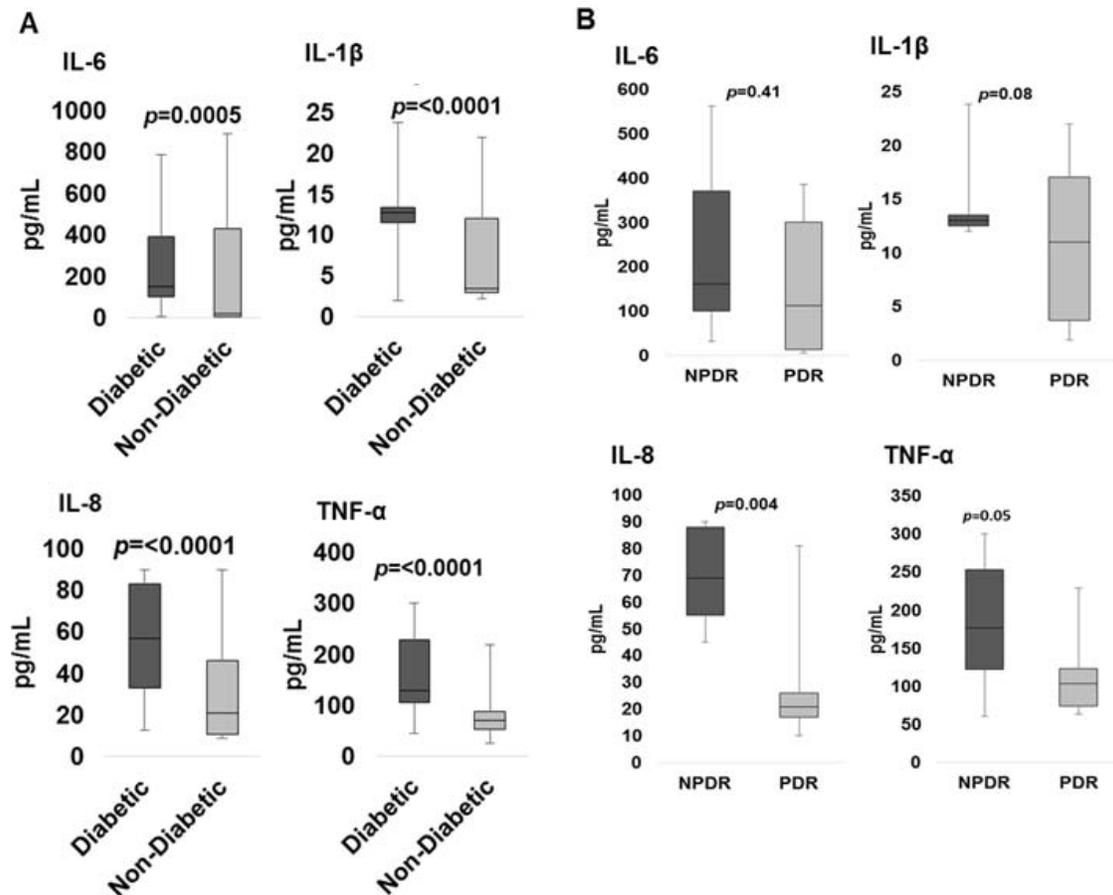


FIGURE 2. Box-and-whisker plot analysis showing comparison of vitreous IL-6, IL-1 β , IL-8, and TNF- α in diabetics ($n = 22$) compared to nondiabetics ($n = 28$) (A) and diabetic patients with NPDR compared to PDR (B).

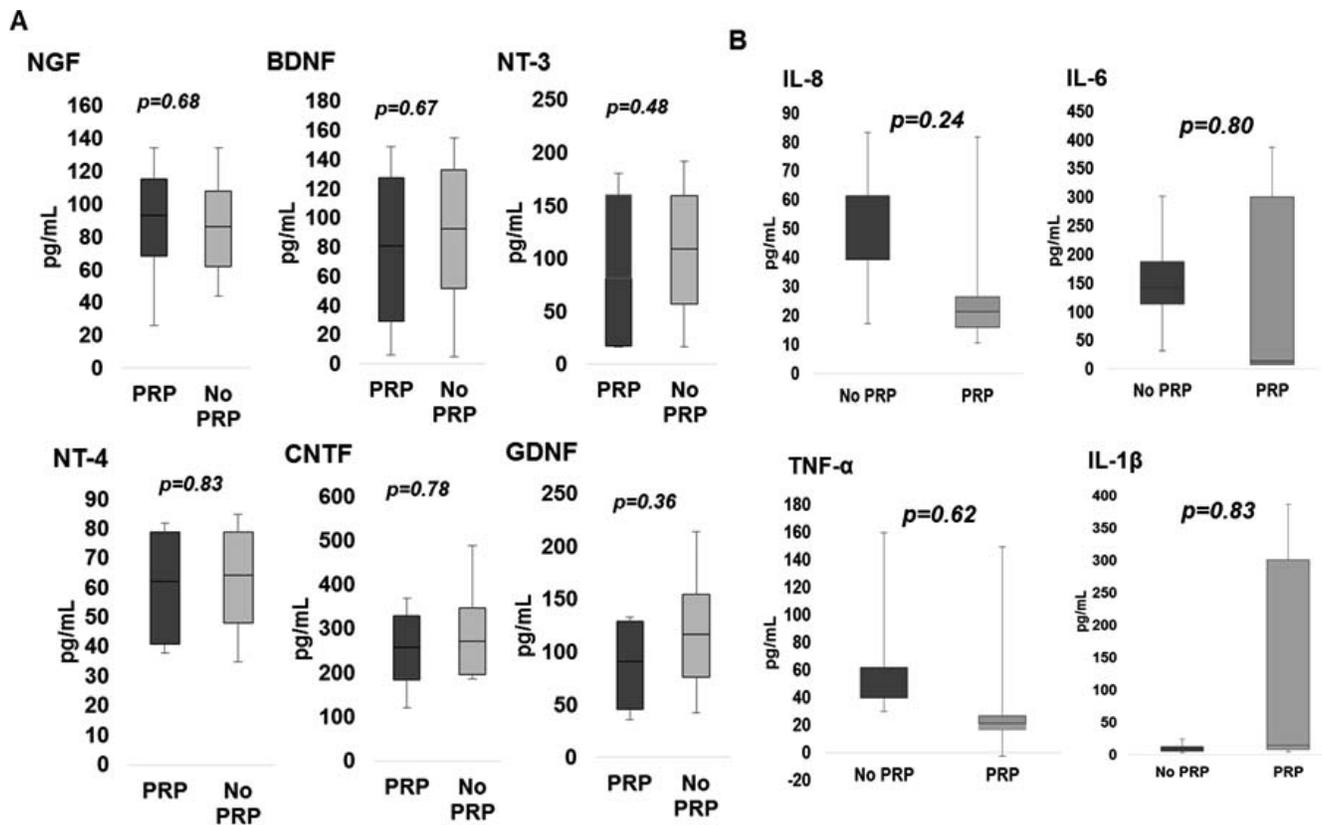


FIGURE 3. Box-and-whisker plot analysis of vitreous neurotrophin (A) and inflammatory cytokine (B) levels in diabetics who have undergone peripheral retinal ablation with PRP compared to diabetics without a history of PRP.

anti-inflammatory cytokine, IL4 (Fig. 4C), and an anti-inflammatory drug, dexamethasone (Fig. 4D). To this end our results indicate that Müller glia were unable to secrete NTs in the conditioned media in response to these anti-inflammatory agents.

These findings indicate that Müller glia could be one of the potential sources of increased NT levels in the vitreous of patients with DR under chronic inflammatory conditions. Since NTs are known to exert neuroprotection, we then investigated whether NTs can diminish inflammation or oxidative stress-induced neuronal cell death, using mouse cone photoreceptor cell line 661W. Our data showed that

GDNF treatment attenuated cell death (TUNEL stain) of 661W cells in response to inflammatory (TNF-α) and oxidative stress (H₂O₂) conditions, as in the case of DR (Fig. 5).

DISCUSSION

DR is a multifactorial disease affecting the retina with an extremely complex pathobiology involving a variety of cell types (both residential and infiltrated), molecules, and factors. From the clinical presentation, DR is broadly classified as NPDR or PDR.²¹ NPDR represents an early stage of the disease in which symptoms are either mild or nonexistent. This progresses to PDR, a more advanced form of the disease where neovascularization is evident in the retina. Because of the severity of PDR, significant research effort has been devoted in determining the pathogenesis of transition from NPDR to PDR and to identify potential biomarkers to aid in early diagnosis, including ETDRS.^{22,23} Clearly, a plethora of studies have implicated inflammation in the pathogenesis of DR, with progression to PDR being strongly correlated with increased inflammatory mediators.²⁴ While inflammation is a protective host response to infection or tissue injury, it must be resolved quickly to prevent collateral tissue damage, especially in the retina.²⁵ How inflammation is initiated and persists in DR or whether it is a cause or effect still remains the key questions in the field. Similarly, how increased inflammatory milieu in DR influences other growth factors, including NTs, is not clearly understood. We postulate that increased inflammatory mediators in DR could trigger the production of protective agents to counterregulate the harmful effects of chronic inflammation and induce neuroprotection. In the current study, we showed that patients

TABLE 3. Quantitative Analysis and Comparison of Intravitreal Levels of Various Neurotrophins in Severe Nonproliferative Diabetic Retinopathy and Proliferative Diabetic Retinopathy Comparing History of Panretinal Photocoagulation

Neurotrophin	History of PRP, Mean, pg/mL (N = 9)	No History of PRP, Mean, pg/mL (N = 6)	P Value
NGF	92.8 (SEM 10.6)	85.8 (SEM 13.3)	0.69
BDNF	80.6 (SEM 17.9)	92.3 (SEM 22.6)	0.69
NT-3	81.2 (SEM 26.1)	108.9 (SEM 26.0)	0.49
NT-4	62.1 (SEM 20.1)	64.3 (SEM 19.4)	0.83
CNTF	258.128 (SEM 26.9)	272.4 (SEM 46.4)	0.78
GDNF	91.0 (SEM 14.6)	115.7 (SEM 24.0)	0.37

pg/ml, picograms/milliliter; NGF nerve growth factor; BDNF brain derived neurotrophic factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; CNTF ciliary neurotrophic factor; GDNF glial cell line-derived neurotrophic factor; PRP, pan-retinal photocoagulation.

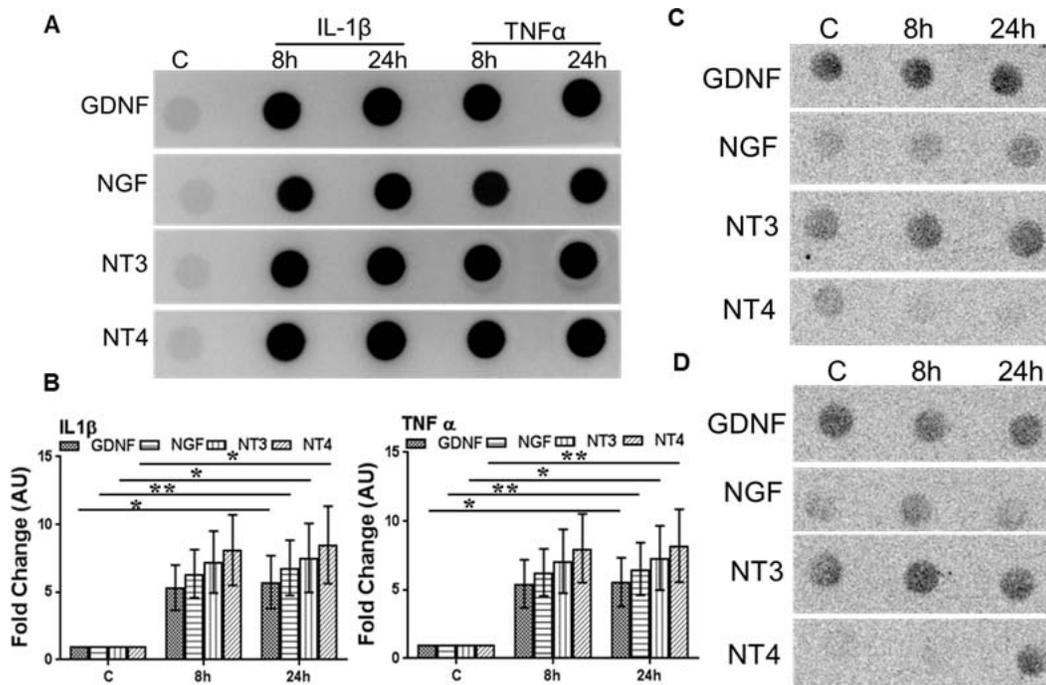


FIGURE 4. Inflammatory cytokines induce NT production in Müller glia. Human retinal Müller glia (MIO-M1 cell line) were challenged with recombinant TNF- α or IL-1 β (100 ng/mL each) (A, B), IL-4 (100 ng/mL) (C), and dexamethasone (100 μ M) (D) for the indicated time points. The secretion of NTs into the conditioned media was detected by dot blot, and the intensities of the dots were quantitated by densitometric analysis, using ImageJ and presented as fold-changes (arbitrary units; AU), using a value of 1 for the control samples (B). Statistical analysis was performed by using a 1-way ANOVA (* $P < 0.01$; ** $P = 0.001$), for comparisons of control versus stimulated cells. Data points and bars represent mean \pm SD of triplicates from three independent experiments.

with DR have high levels of both inflammatory mediators and NTs in their vitreous. Moreover, a comparative analysis revealed higher vitreous inflammatory mediators in NPDR than in PDR. Furthermore, in a proof-of-principle in vitro study, we demonstrated the mechanistic basis of NT production and its potential role in protecting retinal neurons under inflammatory and oxidative stress conditions.

Previous studies analyzing intravitreal inflammatory cytokines in DR have shown a significant increase in levels of IL-6, IL-8, IL-1 β , and TNF- α .^{26–28} These studies, however, have mainly focused on PDR. In the present study, the inflammatory cytokines IL-6, IL-8, IL-1 β , and TNF- α were shown to be significantly elevated in vitreous samples not only of PDR, but also of patients with mild to severe NPDR. Interestingly, levels of cytokines were higher in diabetic eyes with NPDR than with active PDR. Because most (8 of 15) of our PDR patients had vitreous hemorrhage and because of the known compromised blood-retinal barrier in PDR, there is a possibility that serum/plasma proteins might have leaked into the vitreous and diluted the relative amount of NTs and cytokines. However, we did not find a significant difference in average concentration of total vitreous protein in PDR ($4.72 \pm 3.1 \mu\text{g}/\mu\text{L}$) versus NPDR ($4.86 \pm 2.4 \mu\text{g}/\mu\text{L}$) patients. Our findings also corroborated with those of Loukovaara et al.,²⁹ showing no major differences in vitreous protein levels in a large cohort of PDR and NPDR patients. Hence, we concluded that differential levels of inflammatory mediators or NTs, in our study, are unlikely due to altered concentration of total vitreous proteins in PDR. Overall, these results highlight the increased activity of these inflammatory cytokines in the early stages of DR. One of the potential implications of these findings is to therapeutically target inflammatory milieu in NPDR patients to prevent progression to PDR. Indeed, a recent study by Wykoff and colleagues³⁰ supports this notion

whereby intravitreal corticosteroid injections in NPDR eyes have been shown to decrease the rate of progression from NPDR to PDR in patients in FAME A and B trials receiving intravitreal fluocinolone. Additionally, a DRCR.net study called Protocol W is being started in order to compare the reduction in progression from NPDR to PDR when anti-VEGF and PRP laser are applied to severe NPDR patients. Moreover, new Food and Drug Administration (FDA) indications for intravitreal ranibizumab have changed to now incorporate any degree of DR. Hence, an early indication of increased inflammatory mediators in eyes of diabetic patients, as reported in our study, may allow initiating therapeutic strategies to prevent or halt the progression to PDR.

Another unique aspect of our study is the simultaneous assessment of inflammatory mediators and NTs in vitreous of patients with DR. Neurotrophins, released from glial cells such as Müller cells, are a group of functionally and structurally related growth factors that play a critical role in the development, survival, maintenance, and repair of the nervous system, as well as play essential roles in angiogenesis and fibrosis.³¹ Several studies have shown that inflammation alters the expression of neurotrophins. In the brain, administration of proinflammatory cytokines or lipopolysaccharides (LPS) causes a significant reduction in BDNF expression.³² Similarly, BDNF levels are reported to be reduced in DR,³³ whereas GDNF levels are increased.^{14,33} This study provided evidence that NTs are present in the vitreous in both diabetics and nondiabetic patients. Moreover, this human study showed vitreous levels of NTs, including NGF, BDNF, NT-3, NT-4, CNTF, and GDNF, to be statistically significantly elevated in patients with DR when compared to patients without a history of diabetes mellitus. In previous animal studies of NT levels, both NGF and BDNF are undetected in both diabetic and control samples¹³; however,

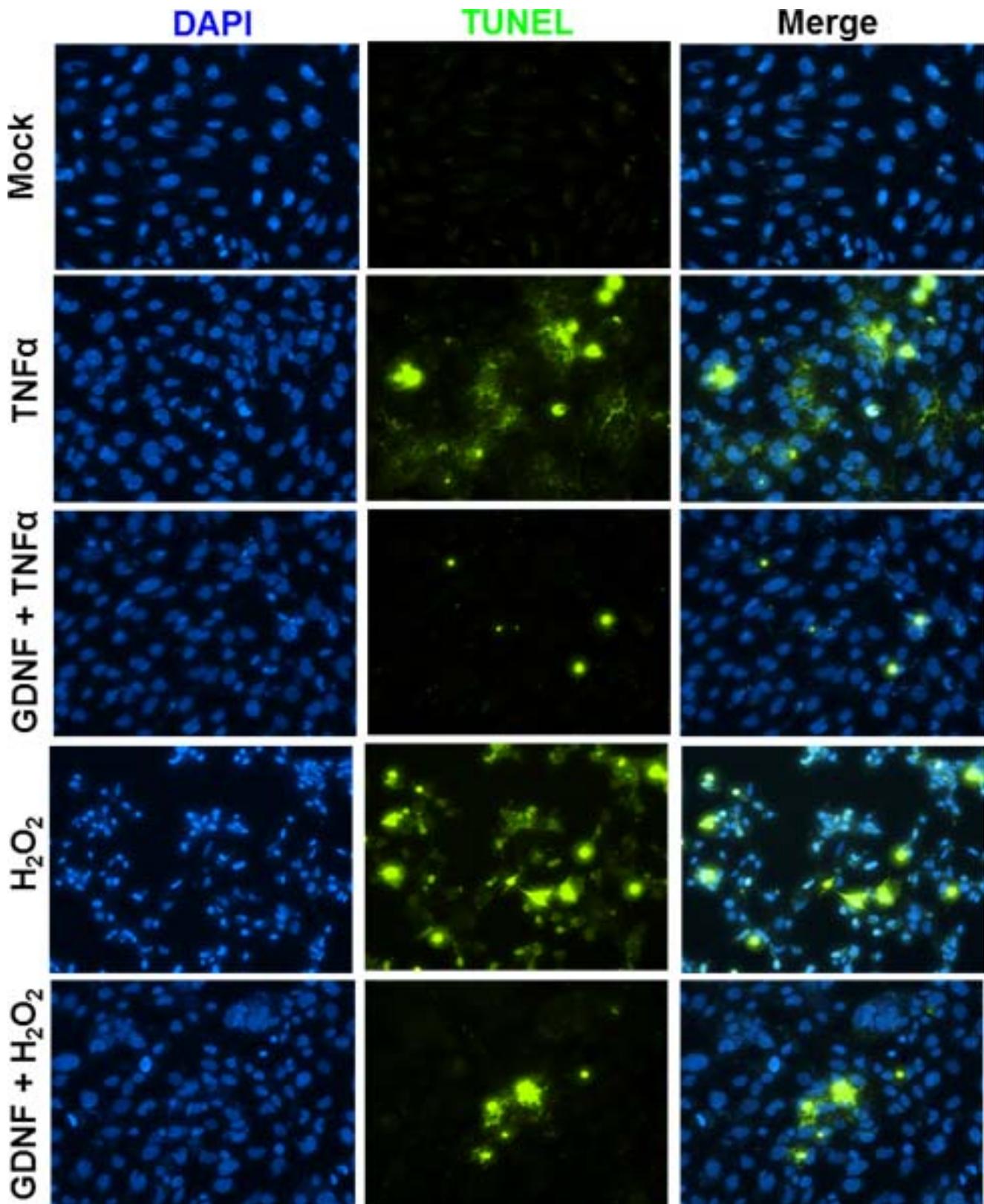


FIGURE 5. GDNF protects oxidative stress and inflammation-induced photoreceptor cell death. Mouse cone photoreceptor cells (661W cell line) were challenged with H_2O_2 (100 μ M) and $TNF-\alpha$ (100 ng/mL) in the presence and absence of GDNF (100 ng/mL) for 24 hours. The neuroprotective effect of GDNF was measured by measuring the cell death by TUNEL staining (*blue*, 4',6-diamidino-2-phenylindole [DAPI] nuclear stain; *green*, TUNEL+ve cells).

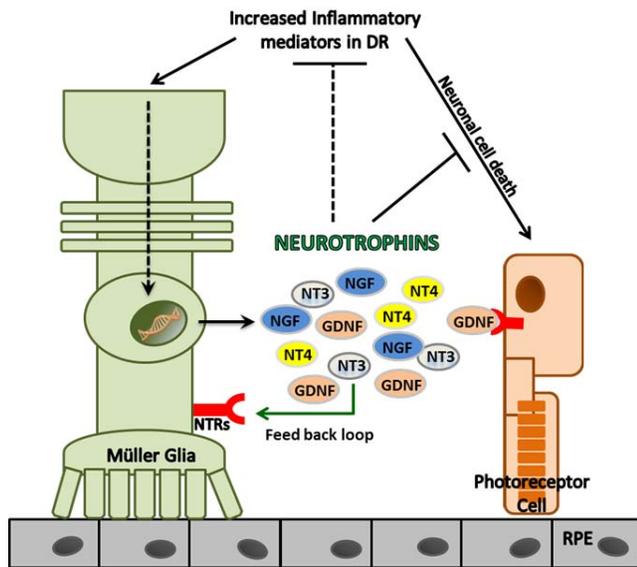


FIGURE 6. Schematic of the relationship between inflammatory mediators and NTs in diabetic retinopathy. Patients with diabetic retinopathy have increased levels of inflammatory mediators in vitreous. This increased inflammatory milieu triggers the production of NTs in retinal cells, such as Müller glia, which in turn exert neuroprotective and anti-inflammatory effects to prevent neuronal cell death in the retina.

our results showed that both NGF and BDNF are not only present in the human vitreous, but also expressed at a higher concentration in diabetics versus nondiabetics.

Ablating the peripheral retina in PDR has been shown to result in regression of undesired neovascularization.³⁴ Our subanalysis of intravitreal levels of NTs in diabetic patients who had a history of PRP, compared to control patients of diabetics with either severe NPDR or PDR who met criteria for PRP but had no history of PRP, did not show a statistically significant difference in local levels of NTs between the two groups. The small sample size limits the interpretation and power of this analysis; however, PRP did not appear to alter NT levels in our study.

NTs signal by binding and activating a dual receptor system consisting of Trks (TrkA, TrkB, and TrkC) and p75NTR. Signaling via Trks mediates the prosurvival effects of NTs on neurons, whereas activation of p75NTR triggers apoptosis.^{35,36} Moreover, p75NTR has recently been shown to be essential in angiogenesis and is upregulated in hypoxic and dystrophic retina conditions, including DR.³⁶⁻³⁹ Hence, NTs exert opposite effects mechanistically depending on which type of receptors they interact with and the target cell types involved.⁴⁰ While we did not aim to estimate NT receptor concentrations in our study, in part because of limited sample availability, previous studies¹³ have shown increased expression of Trk A, TrkB, and p75NTR in the vitreous of DR patients. The increased availability of ligands and NT receptors suggests the possibility of enhanced ligand-receptor interactions and given the known role of NTs in neuroprotection, one would expect that increased NT levels should result in reduced cell death in DR. However, the functional outcome of NT interaction with its respective receptors is difficult to study in humans. While several clinical trials have been performed to treat neurodegenerative diseases by increasing the supply of neurotrophic factors, their clinical use is somewhat limited owing to difficulties in maintaining the optimum levels and their poor pharmacoki-

netics profiles.^{12,36} Currently, the use of neuroprotective agents in DR is an ongoing debate.^{41,42} As DR is now being increasingly accepted as a neurodegenerative disease,^{8,43} the better understanding of NT production and its signaling in the retina may accelerate new therapeutic approaches in the management of DR.

To investigate potential mechanisms of increased NT production in DR (Fig. 6), we postulated that increased inflammatory milieu may potentiate retinal glial cells such as Müller glia to secrete neurotrophic factors as reported in several disease conditions.⁴³⁻⁴⁵ Indeed, our in vitro data showed that TNF- α and IL-1 β stimulation of Müller glia resulted in increased production of NTs, suggesting that Müller glia are one of the potential sources of NTs in the retina. Both increased inflammatory mediators and oxidative stress in DR have been shown to accelerate cell death in a variety of retinal cells. To assess the biological function of NT under these conditions, we challenged cone photoreceptor cells with H₂O₂ and TNF- α in presence of GDNF. Our data showed that GDNF treatment inhibited photoreceptor cell death induced by oxidative stress and inflammation. We have previously reported that infectious stimuli, such as those evoked in bacterial endophthalmitis, trigger the production of NTs in the retina to counterregulate inflammation and induce protection (Kumar and Singla. *IOVS* 2012;53:ARVO E-Abstract 2769). Similarly, exogenous administration of BDNF upregulates anti-inflammatory cytokine IL-10 and reduces the expression of inflammatory cytokine TNF- α in experimental stroke.⁴⁶ Thus, apart from exerting neuroprotective effects, secreted NTs may also counterregulate inflammation in DR (Fig. 6).

In conclusion, to our knowledge, this study is the first to simultaneously assess vitreous levels of inflammatory cytokines and NTs in DR patients. Our comparative analysis of inflammatory mediators in PDR versus NDPR, demonstrating higher levels in NDPR, suggests that early anti-inflammatory therapy may prevent disease progression to PDR. Moreover, our in vitro data provides mechanistic insight into the production and functional role of NTs under inflammatory conditions, evoked in DR.

Acknowledgments

Supported by an unrestricted grant from Research to Prevent Blindness (RPB) to the Department of Ophthalmology/Kresge Eye Institute at Wayne State University, Detroit, Michigan, United States. The immunology resource core is supported by a National Institutes of Health Center Grant P30EY004068.

Disclosure: **J.D. Boss**, None; **P.K. Singh**, None; **H.K. Pandya**, None; **J. Tosi**, None; **C. Kim**, None; **A. Tewari**, None; **M.S. Juzych**, None; **G.W. Abrams**, None; **A. Kumar**, None

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