Neurodegenerative and Inflammatory Pathway Components Linked to TNF- α /TNFR1 Signaling in the Glaucomatous Human Retina

Xiangjun Yang,¹ Cheng Luo,¹ Jian Cai,² David W. Powell,^{3,4} Dahai Yu,⁵ Markus H. Kuehn,⁵ and Gülgün Tezel^{1,6}

PURPOSE. This study aimed to determine retinal proteomic alterations in human glaucoma, with particular focus on links to TNF- α /TNFR1 signaling.

METHODS. Human retinal protein samples were obtained from 20 donors with ($n = 10$) or without ($n = 10$) glaucoma. Alterations in protein expression were individually analyzed by quantitative LC-MS/MS. Quantitative Western blot analysis with cleavage or phosphorylation site-specific antibodies was used for data validation, and cellular localization of selected proteins was determined by immunohistochemical analysis of the retina in an additional group of glaucomatous human donor eyes ($n = 38$) and nonglaucomatous controls ($n = 30$).

RESULTS. Upregulated retinal proteins in human glaucoma included a number of downstream adaptor/interacting proteins and protein kinases involved in TNF- α /TNFR1 signaling. Bioinformatic analysis of the high-throughput data established extended networks of diverse functional interactions with death-promoting and survival-promoting pathways and mediation of immune response. Upregulated pathways included death receptor-mediated caspase cascade, mitochondrial dysfunction, endoplasmic reticulum stress, calpains leading to apoptotic cell death, NF- κ B and JAK/STAT pathways, and inflammasome-assembly mediating inflammation. Interestingly, retinal expression pattern of a regulator molecule, TNFAIP3, exhibited prominent variability between individual samples, and methylation of cytosine nucleotides in the *TNFAIP3* promoter was found to be correlated with this variability among glaucomatous donors.

CONCLUSIONS. Findings of this study reveal a number of proteins upregulated in the glaucomatous human retina that exhibit many links to TNF- α /TNFR1 signaling. By highlighting various signaling molecules and regulators involved in cell death and immune response pathways and by correlating proteomic findings with epigenetic alterations, these findings provide a frame-

work motivating further research. (*Invest Ophthalmol Vis Sci.* 2011;52:8442–8454) DOI:10.1167/iovs.11-8152

The prevailing view is that glaucoma pathogenesis is multifactorial, with a complex interplay of elevated intraocular pressure-induced events and genetic/epigenetic/aging-related susceptibility factors contributing to neurodegeneration. Glial activation response and secondary inflammatory/autoimmune processes are also regarded as continuous components of glaucomatous neurodegeneration. It is widely accepted that chronic activation of glial cells and accompanying increases in the production of proinflammatory cytokines, primarily including TNF- α , are hallmarks of inflammation/parainflammation in glaucomatous tissue, although a cause-effect relationship remains to be validated.^{1,2} TNF- α , with beneficial and neurotoxic effects in the central nervous system (CNS) along with key physiological functions in the maintenance of immune homeostasis, has been implicated in the pathogenesis of a wide spectrum of human neurodegenerative diseases. It is also increasingly evident that TNF- α through the binding of TNFR1, a death receptor, exhibits important links to glial activation response, mediation of retinal ganglion cell (RGC) death, and inflammatory processes during the neurodegenerative injury in glaucoma.³

Despite growing evidence that supports important roles of TNF- α in glaucomatous neurodegeneration, opposing consequences of TNF- α signaling make it difficult to exploit for neuroprotective strategies. Respecting the diverse bioactivities of this multifunctional cytokine, molecular dissection of specific signaling components can provide the possibility to specifically inhibit RGC death or modulate immune response without compromising survival-promoting signals. To better understand molecular components of the neurodegenerative signaling in human glaucoma, this study analyzed retinal protein samples obtained from donor eyes with or without glaucoma. Findings of this comparative analysis supported a prominent upregulation of TNF- α /TNFR1 signaling in the glaucomatous human retina. By highlighting various signaling molecules and regulators involved in cell death and immune response pathways in human glaucoma, these findings provide framework information and motivate further research.

MATERIALS AND METHODS

Donor Eyes

Retinal protein samples obtained from 10 human donor eyes with glaucoma (age, 84.7 ± 8) and 10 eyes without glaucoma (age, 83.7 ± 7) were individually analyzed by capillary liquid chromatography coupled with linear ion trap mass spectrometry (LC-MS/MS). As previously described,^{4,5} retinal tissue punches were collected within <6 hours after death, and glaucomatous eyes were well documented.

From the Departments of ¹Ophthalmology and Visual Sciences, ²Pharmacology and Toxicology, ³Medicine, ⁴Biochemistry & Molecular Biology, and ⁵Anatomical Sciences & Neurobiology, University of Louisville School of Medicine, Louisville, Kentucky; and ⁶Department of Ophthalmology & Visual Sciences, University of Iowa, Iowa City, Iowa.

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Corresponding author: Gülgün Tezel, University of Louisville School of Medicine, Kentucky Lions Eye Center, 301 E. Muhammad Ali Boulevard, Louisville, KY 40202; gulgun.tezel@louisville.edu.

In addition, cellular localization of selected proteins was determined by immunohistochemical analysis of retinal tissue sections obtained from an additional group of glaucomatous and nonglaucomatous human donor eyes. This group included 38 donor eyes with a diagnosis of glaucoma (age, 76.8 ± 11) and 30 eyes without glaucoma (age, 71.0 ± 15), all fixed within 12 hours after death. Detailed information on donor demographics and clinical data has been previously published.⁶ All the human donor eyes were handled according to the tenets of the Declaration of Helsinki.

Proteomic Analysis

Protein samples prepared with a lysis buffer containing 50 mM Hepes-KOH pH 8.0, 100 mM KCl, 2 mM EDTA, 0.10% NP-40, 2 mM dithiothreitol, 10% glycerol, and protease and phosphatase inhibitors were analyzed by label-free quantitative LC-MS/MS, as previously described.⁷ Briefly, trypsin-digested samples were loaded onto an analytical 2D capillary chromatography column packed with strong cation exchange (SCX) and C₁₈ reversed-phase (RP) resin (Phenomenex, Torrance, CA). This biphasic column was attached to an analytical RP chromatography column with an integrated, laser-pulled emitter tip. Peptides were eluted from SCX with seven-step gradients of 5%, 10%, 15%, 30%, 50%, 70%, and 100% of 500 mM ammonium acetate and eluted into a linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) according to a linear HPLC gradient (20-minute 0% B, 80-minute 40% B, and 90-minute 60% B at a flow rate of 200 nL/min with mobile phase-A 5% acetonitrile/0.1% formic acid and mobile phase-B 80% acetonitrile/0.1% formic acid). Protein identification from MS/MS spectra was performed with proteomics analysis software (Sequest Sorcerer; Sage-N Research, San Jose, CA), which was set up to search a FASTA formatted human protein database (Human RefSeq) with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.2 Da. Meta-analysis software (Scaffold; Proteome Software Inc., Portland, OR) was used to validate peptide and protein identifications based on the criteria of 95.0% and 99.0% probability and at least two peptides as specified by the Prophet peptide⁸ and protein algorithms.⁹ The abundance of each identified protein was determined by normalizing the number of unique spectral counts matching to the protein by its predicted molecular weight.¹⁰ The Mann-Whitney rank sum test was used to determine the significant difference in protein expression between glaucomatous and control samples.

As in previous studies,^{4,5,11} we used pathway analysis software (Ingenuity Pathways Analysis [IPA]; Ingenuity Systems, Mountain View, CA) for bioinformatic analysis. Our high-throughput dataset and the corresponding expression values uploaded into the application were analyzed to define functional patterns and generate extended interaction networks using the database (Ingenuity Pathway Knowledge Base; Ingenuity Sys-

tems). Canonical pathway analysis identified the pathways from the IPA library that were most significant to the dataset by the right-tailed Fisher's exact test.

Western Blot Analysis

Immunoblotting used primary antibodies to cleaved caspase 1 (1:1000; Millipore, Billerica, MA), caspases 3 and 8 (1:1000; Cell Signaling, Danvers, MA), caspases 9 and 12 (1:500; Abcam, Cambridge, MA), nuclear factor kappa B (NF-κB) subunits, p50 [phospho-Ser932] (1:500; GenWay, San Diego, CA) and p65 [phospho-Ser276] (1:1000; Cell Signaling), signal transducers and activators of transcription (STAT)1 [phospho-Tyr701], STAT2 [phospho-Tyr690], STAT3 [phospho-Ser727], STAT4 [phospho-Tyr693], STAT5 [phospho-Tyr694], STAT6 [phospho-Tyr641], (1:1000; Cell Signaling), and TNF-α-induced protein 3 (TNFAIP3; 1:1000; Epitomics, Burlingame, CA). In addition, a β-actin antibody (Sigma-Aldrich, St. Louis, MO) was used to reprobe immunoblots for loading and transfer control. The secondary antibody incubation used a specific IgG conjugated with horseradish peroxidase (1:2000; Sigma-Aldrich). The primary antibody was omitted to provide negative control. After normalization to β-actin, the average band intensity value obtained from nonglaucomatous samples was used to calculate the fold change in protein expression in glaucomatous samples.

Morphologic Analysis

Double immunofluorescence labeling used the same primary antibodies described for Western blot analysis (1:100). In addition, antibodies against Brn-3 (not subtype selective; 1:200) or glial fibrillary acidic protein (GFAP; 1:200), both from Santa Cruz Biotechnology (Santa Cruz, CA), were used to identify RGCs and astrocytes. A mixture of Alexa Fluor 488- or 568-conjugated species-specific IgGs (1:400; Molecular Probes, Eugene, OR) was used for the secondary antibody incubation. Negative controls were performed by replacing the primary antibody with serum or by using an inappropriate secondary antibody to determine species specificity.

SNP Analysis

Genomic DNA was isolated from retinal tissue samples of 5 donors with glaucoma using a purification kit (DNeasy Blood & Tissue Kit; Qiagen, Hilden, Germany). All fragments were amplified using polymerase (Gotaq Flexi; Promega, Madison, WI) and were sequenced (DNA Analyzer; Applied Biosystems, Foster City, CA). Primer sequences used for amplification and sequencing are provided in Table 1A.

TABLE 1. Primer Sequences Used for Amplification and Sequencing

A. SNP Analysis

rs112229105	F	GAAAGCTGTGAAGATACGGGAGAGA
	R	CCCCGAATAGAGATTCTATATAAAGTCTC
rs5029941/rs2230926	F	GCTGTCATCATCTTGTGAAATATCAGTTTG
	R	GGAGGTTTCTGGTGTTCCTTCCATTGAG
rs61756235	F	CAGGTAACAGAGTTCAATGGAATTTGTAGA
	R	CTCTTACTAACCAAGCAAGTCACAGAAC
rs34935799	F	GGGTGATCATTGAATGATGGTTTCATG
	R	ATTCCAAACCTCTTAGCATTTTGCTCTGTTT
rs3734553/rs5029957	F	GTCCCAACAGAGAGAGCCAGG
	R	CTCCTGCTCAGACACCCTTAAGC

B. Methylation Sequencing

Outer primers for first round	F	GATTTAGAGAGTTACGTGATTTTGAAAG
	R	TCCAACAAACTCCCAATCCAAAAAC
Inner primers for second round	F	GTATATAATTGAAACGGGGTAAAGTAGATTG
	R	ATACCAACCCGAAAATCGCTACCCAACATACACC

F, forward; R, reverse.

Methylation Sequencing

Genomic DNA extracted from retinal tissue samples of 5 donors with glaucoma was subjected to bisulfite treatment (BisulFlash DNA Modification Kit; Epigentek, Farmingdale, NY). After conversion, the promoter region was amplified by nested PCR using DNA polymerase (Platinum *Taq* Hi-Fi; Invitrogen, Carlsbad, CA) and was sequenced as described. Primer pairs surrounding the CpG island within the *TNFAIP3* promoter were designed using the MethPrimer online tool.¹² Primer sequences used for amplification and sequencing are provided in Table 1B.

RESULTS

Quantitative LC-MS/MS analysis of human retinal protein samples resulted in the identification of hundreds of proteins with high confidence that exhibited upregulated or downregulated expression in glaucomatous samples. Bioinformatic analysis identified the pathways from the IPA library that were most significantly associated with our high-throughput data. Top canonical pathways most significant to our dataset included death receptor signaling pathway (right-tailed Fisher's exact test; $P < 0.05$). Here, we present the upregulated proteins exhibiting links to TNF- α /TNFR1 signaling.

As listed in Table 2, upregulated retinal proteins in human glaucoma included TNFR1 and a number of downstream adaptor/interacting proteins, such as TNFR1-associated death domain protein (TRADD), mitogen-activated protein kinase (MAPK)-activating death domain-containing protein, different members of the TNFR-associated factor (TRAF) family, and NF- κ B. Identified proteins also included various regulator molecules involved in TNFR signaling, such as caspase 8 and FADD-like apoptosis regulator (CFLAR, also called FLICE-inhibitory protein) and optineurin. Another regulator protein we detected was TNFAIP3, also known as A20, which is a potent inhibitor of NF- κ B activation and a negative regulator of TNF- α signaling leading to apoptosis and inflammation. Despite an overall prominent difference between glaucoma-

tous and nonglaucomatous samples, glaucomatous samples exhibited individual differences in increased expression of different proteins. However, the presented data were consistent in at least 6 of 10 glaucomatous samples for each of the listed proteins, except for the regulator proteins, mainly including TNFAIP3. Interestingly, the expression of this protein exhibited prominent individual differences.

As shown in Table 3, we detected the upregulation of a number of protein kinases specific to TNFR signaling, such as receptor-interacting serine-threonine kinase 1 (RIPK), NF- κ B-inducing kinase (NIK), and inhibitory kappa B (κ B) kinases (κ Bs) leading to NF- κ B activation. In addition, we detected the upregulation of various other protein kinases in glaucomatous samples that are also linked to TNFR1 signaling. As listed in Table 3, these kinases included numerous members of MAPKs (including the apoptotic c-Jun N-terminal kinase [JNK]) and janus kinases (JAKs).

Table 4 shows the proteins linked to apoptosis signaling in the glaucomatous human retina, which included caspases, such as caspase 8 (an early caspase in receptor-mediated pathway) and caspase 9 (related to mitochondrial pathway). Our data also supported the increased expression of various members of the Bcl-2 family controlling the mitochondrial cell death pathway. These included the upregulation of proapoptotic Bax (along with Bid and Bim, which were detectable only in glaucomatous samples) and antiapoptotic Bcl-XL. In addition, we detected the upregulation of various mitochondrial proteins related to the mitochondrial pathway of apoptosis, such as apoptosis-inducing factor and endonuclease G (Table 4). Also detectable was a prominent upregulation of various calcium-dependent cysteine proteases (calpains), another group of cell death mediators, in glaucomatous samples. Caspase recruitment domain-containing proteins that were also detectable in the human retina function in the regulation of both apoptosis and inflammatory responses. Many endoplasmic reticulum (ER)-resident proteins were upregulated in glaucomatous sam-

TABLE 2. Analysis of Retinal Protein Expression in Human Glaucoma: Proteins Involved in the TNFR Signaling

Symbol	Protein Name	RefSeq	Fold Change
TNF- α	Tumor necrosis factor- α	NP_000585	3.1*
TNFRSF1A (TNFR1)	Tumor necrosis factor receptor superfamily, member 1A	NP_001056	2.1*
TRADD	TNFRSF1A-associated death domain-containing protein	NP_003780	2.3*
MADD	MAP kinase-activating death domain-containing protein isoform a	NP_569826	1.9*
MADD	MAP kinase-activating death domain-containing protein isoform g	NP_569831	1.1
CFLAR (FLIP)	Caspase 8 and FADD-like apoptosis regulator	NP_003870	1.2
TRAF1	TNFR-associated factor 1	NP_005649	2.4*
TRAF2	TNFR-associated factor 2	NP_066961	3.6*
TRAF3IP1	TNFR-associated factor 3 interacting protein 1	NP_056465	1.3
TRAF4	TNFR-associated factor 4	NP_004286	1.5
TRAF4 variant	TNFR-associated factor 4 isoform 2	NP_665694	1.9*
TFAF2 (SNX6)	TNFR-associated factor 4-associated factor 2	NP_067072	1.8*
TRAF5	TNFR-associated factor 5	NP_004610	1.4
TRAF6	TNFR-associated factor 6	NP_665802	2.8*
TRAP2	TNFR-associated protein 2	NP_002799	1.1
TRIP	TNFR-associated factor interacting protein	NP_005870	1.2
TIFA	TRAF-interacting protein with FHA domain-containing protein A	NP_443096	1.6
KCTD13	TNF- α -induced protein 1-like adaptor protein	NP_849194	2.1*
NF- κ B	Nuclear factor-kappa B subunit p105 (NFKB1/p50)	NP_003989	1.9*
I κ BE	Nuclear factor-kappa B inhibitor epsilon	NP_004547	1.4
TNFAIP3 (A20)	TNF- α -induced protein 3	NP_006281	1.2
TNIP1	TNF- α -induced protein 3-interacting protein 1	NP_006049	2.5*
TNIP2	TNF- α -induced protein 3-interacting protein 2	NP_077285	1.3
OPTN	NEMO-Related Protein (NRP), optineurin	NP_068815	1.2

Retinal protein samples obtained from human donor eyes with ($n = 10$) or without ($n = 10$) glaucoma were individually analyzed by quantitative LC-MS/MS. All listed proteins were identified with high confidence (greater than 99.0% probability assigned by the Protein Prophet algorithm) based on at least two identified peptides.

* Significant difference in protein expression between glaucomatous and control samples (Mann-Whitney rank sum test; $P < 0.05$).

TABLE 3. Analysis of Retinal Protein Expression in Human Glaucoma: Selected Protein Kinases

Symbol	Protein Name	RefSeq	Fold Change
IKKE	Inhibitor of kappa-B kinase epsilon	NP_054721	1.9*
IKKG (NEMO)	NF- κ B essential modulator, inhibitor of kappa-B kinase gamma	NP_003630	3.4*
RIPK1	Receptor (TNFRSF)-interacting serine/threonine kinase 1	NP_003795	2.2*
RIPK2 (CARD3)	Receptor-interacting serine/threonine kinase 2	NP_003812	1.1
ROCK1	Rho-associated protein kinase 1	NP_005397	2.3*
RAF1 (c-Raf)	Raf proto-oncogene serine/threonine-protein kinase 1	NP_002871	1.4
STK17A	Serine/threonine kinase 17a (apoptosis-inducing)	NP_004751	1.9*
STK25	Serine/threonine kinase 25 (oxidant stress response kinase 1)	NP_006365	1.6
MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1 isoform 2	NP_009112	1.2
MAP4K2 (GCK)	Mitogen-activated protein kinase kinase kinase kinase 2	NP_004570	6.4*
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	NP_004825	1.1
MAP3K3 (MEKK3)	Mitogen-activated protein kinase kinase kinase 3	NP_002392	1.2
MAP3K4 (MEKK4)	Mitogen-activated protein kinase kinase kinase 4	NP_005913	1.1
MAP3K5 (MEKK5, ASK1)	Mitogen-activated protein kinase kinase kinase 5	NP_005914	2.2*
MAP3K6 (MAKK6, ASK2)	Mitogen-activated protein kinase kinase kinase 6	NP_004663	1.3
MAP3K7 (MEKK7)	Mitogen-activated protein kinase kinase kinase 7	NP_663304	1.7
MAP3K14 (NIK)	Mitogen-activated protein kinase kinase kinase 14	NP_003945	2.3*
MAP3K18 (TAOK3)	Mitogen-activated protein kinase kinase kinase 18	NP_057365	2.4*
MAP2K2 (MKK2, MEK2)	Mitogen-activated protein kinase kinase 2	NP_109587	1.5
MAP2K6 (MKK6, MEK6)	Mitogen-activated protein kinase kinase 6	NP_002749	2.8*
MAP2K7 (MKK7)	Mitogen-activated protein kinase kinase 7	NP_660186	1.4
MAPK1 (ERK2)	Mitogen-activated protein kinase 1 (variant 1)	NP_002736	4.4*
MAPK1 (ERK2)	Mitogen-activated protein kinase 1 (variant 2)	NP_620407	1.3
MAPK3 (ERK1)	Mitogen-activated protein kinase 3	NP_002737	3.0*
MAPK7	Mitogen-activated protein kinase 7	NP_002740	1.5
MAPK8 (JNK1)	Mitogen-activated protein kinase 8	NP_620634	2.9*
JIP2	Mitogen-activated protein kinase 8 interacting protein 2	NP_036456	1.1
MAPK10 (JNK3)	Mitogen-activated protein kinase 10 isoform 3	NP_620446	1.1
MAPK13 (p38d)	Mitogen-activated protein kinase 13	NP_002745	1.3
MAPKAPK5	Mitogen-activated protein kinase-activated protein kinase 5	NP_003659	1.2
PAK2	P21-activated kinase 2	NP_002568	2.6*
PAK 5/7	P21-activated kinase 5/7	NP_065074	1.8
JAK2	Tyrosine-protein kinase Janus kinase 2	NP_004963	1.5
JAK3	Tyrosine-protein kinase Janus kinase 3	NP_000206	1.7

Retinal protein samples obtained from human donor eyes with ($n = 10$) or without ($n = 10$) glaucoma were individually analyzed by quantitative LC-MS/MS. All listed proteins were identified with high confidence (greater than 99.0% probability assigned by the Protein Prophet algorithm) based on at least two identified peptides.

* Significant difference in protein expression between glaucomatous and control samples (Mann-Whitney rank sum test; $P < 0.05$).

ples (Table 4). These included activating transcription factor 6, 78-kDa glucose-regulated protein, and serine/threonine-protein kinase/endoribonuclease inositol-requiring 1.

In addition to many cell death-promoting proteins, our proteomic data supported a prominent upregulation of various proteins involved in intrinsic adaptive/protective mechanisms, such as inhibitor-of-apoptosis proteins (IAPs), heat shock proteins (HSPs), and a number of antioxidants (data not shown).

Besides increased protein expression supporting NF- κ B activation in the glaucomatous human retina, many other proteins linked to inflammatory pathways were upregulated in glaucomatous samples. These included various inflammasome components, including caspase 1, an inflammatory caspase (Table 5).

Bioinformatic analysis of the quantitative data established extended functional networks of the identified proteins with links to death-promoting and survival-promoting pathways of the TNF- α /TNFR1 signaling. Figure 1 shows a simplified version of the protein interaction network generated by IPA.

Western blot analysis and immunohistochemistry using specific antibodies to selected proteins validated increased protein expression/activation and cellular localization. To validate caspase activation during glaucomatous neurodegeneration in human eyes, we subjected our retinal protein samples to Western blot analysis and analyzed tissue sections by immunohistochemistry using cleavage site-specific antibodies. Western blot analysis supported caspase activation by protein cleavage in glaucomatous samples (Fig. 2). Based on immunohistochemical analysis, active caspases were detectable only in the inner retina of glaucomatous

eyes. As shown in Figure 2, double immunofluorescence labeling of the glaucomatous human retina demonstrated prominent localization of cleaved caspases in RGCs.

Quantitative LC-MS/MS analysis detected the upregulation of NF- κ B subunit p50. Western blot analysis using phosphorylation site-specific antibodies showed significantly increased phosphorylation of this subunit and of another subunit (p65) in glaucomatous samples. Based on double-immunofluorescence labeling, phospho-p50 and phospho-p65 were predominantly localized to GFAP-positive astrocytes in the glaucomatous human retina (Fig. 3).

Western blot analysis using phosphorylation site-specific antibodies also supported a significant increase in STAT activation by phosphorylation in glaucomatous samples, and these signaling molecules were localized to RGCs and astrocytes (Fig. 4). We also detected some negative regulators of JAK/STAT signaling in glaucomatous samples, such as feedback inhibitor suppressor of cytokine signaling and protein inhibitors of activated STAT (data not shown).

Western blot analysis also aimed to further validate individual differences in the expression of regulator molecules. However, because of the paucity of our glaucomatous donor tissues, comparative analysis of glaucomatous and nonglaucomatous human samples determined only the expression of TNFAIP3 among various regulator proteins. This analysis revealed a greater than two-fold increase in TNFAIP3 expression in 4 of 10 glaucomatous samples; however, TNFAIP3 expression was unchanged or decreased in the rest of the glaucomatous samples (Fig. 5). As shown

TABLE 4. Analysis of Retinal Protein Expression in Human Glaucoma: Proteins Linked to Apoptosis

Symbol	Protein Name	RefSeq	Fold Change
BCL(X)L	Bcl2-like 1	NP_612815	1.9*
BAX	Bcl2-associated × protein	NP_620116	4.3*
BCL2L2	Bcl2-like 2	NP_004041	1.6
BCL2L10	Bcl2-like 10	NP_065129	1.4
BIM	Bcl2-like 11	NP_619527	†
BCL2L13	Bcl2-like 13	NP_056182	1.1
BID	BH3 interacting domain-containing death agonist	NP_932070	†
ASPP1	Apoptosis-stimulating protein of p53, 1	NP_056131	1.2
TP53BP1	P53-binding protein 1	NP_005648	1.3
CYTC	Cytochrome c, somatic	NP_061820	1.2
AIFM1	Apoptosis-inducing factor 1	NP_665811	2.9*
AIFM2	Apoptosis-inducing factor 2	NP_116186	1.6
ENDOG	Endonuclease G	NP_004426	3.0*
APAF1	Apoptotic peptidase activating factor 1	NP_863651	1.3
CASP8	Caspase 8	NP_001219	1.7
CASP9	Caspase 9	NP_001220	2.1*
CASP10	Caspase 10	NP_001221	1.4
BCL10	Caspase recruitment domain-containing apoptotic signaling protein	NP_003912	2.1*
CARD7 (NALP1)	Caspase recruitment domain-containing family member 7	NP_127497	1.1
CARD8	Caspase recruitment domain-containing family member 8	NP_055774	1.6
CARD9	Caspase recruitment domain-containing protein member 9	NP_434700	1.4
CARD10 (BIMP1)	Caspase recruitment domain-containing family member 10	NP_055365	1.7
CARD11	Caspase recruitment domain-containing family member 11	NP_115791	1.1
CARD12 (IPAF) (NLRC4)	Caspase recruitment domain-containing protein member 12	NP_067032	1.2
PDCD10	Programmed cell death 10; apoptosis-related protein 15	NP_009148	2.7*
THAP1	THAP domain-containing nuclear proapoptotic factor	NP_060575	2.0*
PAR4	Prostate apoptosis response protein 4	NP_002574	2.2*
ACIN1	Apoptotic chromatin condensation inducer 1	NP_055792	1.3
DFFA (ICAD)	DNA fragmentation factor alpha	NP_004392	2.7*
CAPNS1	Calpain small subunit 1	NP_001740	3.8*
CAPN2	Calpain 2 catalytic subunit	NP_001739	1.7
CAPN6	Calpain 6	NP_055104	1.8*
CAPN9	Calpain 9	NP_006606	1.5
CAPN10	Calpain 10	NP_075571	2.0*
CAST	Calpastatin isoform c, calpain inhibitor	NP_775084	2.3*
GRP78 (BIP)	78 kDa glucose-regulated protein, heat shock 70kDa protein 5	NP_005338	5.4*
DNAJC10	DnaJ (Hsp40) homolog C 10	NP_061854	2.2*
ERO1LB	Endoplasmic reticulum oxidoreductin 1-like beta	NP_063944	2.8*
PDI	Protein disulfide isomerase A1	NP_000909	1.4
PDIA6	Protein disulfide isomerase A6 (ERP5)	NP_005733	3.6*
ERP29	Endoplasmic reticulum protein 29	NP_006808	3.3*
ERN1	Serine/threonine-protein kinase/endoribonuclease IRE1	NP_001424	2.5*
ATF6	Activating transcription factor 6	NP_031374	2.7*

Retinal protein samples obtained from human donor eyes with ($n = 10$) or without ($n = 10$) glaucoma were individually analyzed by quantitative LC-MS/MS. All listed proteins were identified with high confidence (greater than 99.0% probability assigned by the Protein Prophet algorithm) based on at least two identified peptides.

* Significant difference in protein expression between glaucomatous and control samples (Mann-Whitney rank sum test; $P < 0.05$).

† Proteins detectable only in glaucomatous samples.

in Figure 5, immunohistochemical analysis of the human retina demonstrated localization of this regulator protein in both Brn-3-positive RGCs and GFAP-positive astroglia. Individual dif-

ferences were detectable in retinal TNFAIP3 immunolabeling among glaucomatous donors. However, unlike quantitative LC/MS/MS and Western blot analyses, immunohistochemical anal-

TABLE 5. Analysis of Retinal Protein Expression in Human Glaucoma: Proteins Involved in the Inflammasome

Symbol	Protein Name	RefSeq	Fold Change
NOD1 (CARD4)	Nucleotide-binding oligomerization domain-containing protein 1	NP_006083	2.1*
NOD2 (CARD15)	Nucleotide-binding oligomerization domain-containing protein 2	NP_071445	1.7
NLRC5	Nucleotide-binding oligomerization domain-containing protein 27	NP_115582	1.4
NLRP3 (NALP3)	NACHT, LRR and PYD domains-containing protein 3 isoform b	NP_899632	2.2*
CASP1	Caspase 1 isoform alpha	NP_150634	3.1*

Retinal protein samples obtained from human donor eyes with ($n = 10$) or without glaucoma ($n = 10$) were individually analyzed by quantitative LC-MS/MS. All listed proteins were identified with high confidence (greater than 99.0% probability assigned by the Protein Prophet algorithm) based on at least two identified peptides.

* Significant difference in protein expression between glaucomatous and control samples (Mann-Whitney rank sum test; $P < 0.05$).

FIGURE 1. Bioinformatic analysis of the comparative proteomic data. IPA established extended functional networks of the identified proteins with links to death-promoting and survival-promoting pathways of the TNF- α /TNFR1 signaling. Shown is a simplified version of the protein interactions network, in which proteins shown in *red* exhibited significantly increased expression and proteins shown in *yellow* exhibited no significant increased expression in glaucomatous samples compared with nonglaucomatous control samples. TNFAIP3 expression, shown in *blue*, exhibited prominent individual differences. Proteins shown in *white* were not detectable by quantitative LC-MS/MS analysis (detailed information for abbreviated proteins is available at www.ncbi.nlm.nih.gov/protein).

ysis primarily determined the cellular localization of selected proteins rather than the quantification of protein extend.

To determine whether genomic variation between donors is correlated with the observed differences in protein expression, we investigated the genomic sequence of *TNFAIP3* by direct sequencing of PCR-amplified exon sequences, with particular attention directed toward several known SNPs (rs112229105, rs5029941, rs2230926, rs61756235, rs3734553, rs5029957, rs34935799). No variation was found in these sites between the donors demonstrating high or low expression levels of TNFAIP3 (data not shown). We also investigated the methylation pattern in the promoter region of *TNFAIP3* using bisulfite sequence analysis. DNA was extracted from the retinal tissue obtained from 3 glaucomatous donors with low protein expression and 2 glaucomatous donors with high protein expression. As shown in Figure 6, our data indicated that one CpG site (-135) within the promoter region and two CpG sites in the first intron were consistently methylated in donors with low TNFAIP3 expression, whereas methylation was not ob-

served in donors with high expression. Furthermore, we detected a very consistent methylation pattern of non-CpG sites. Cytosine residues at positions -173, -144, -114, -96, -66, -4, and -2 were consistently methylated in donors with low TNFAIP3 expression (Fig. 6A). Interestingly, our data did not indicate partial conversion of cytosine residues, but all cytosine residues in these positions were either completely methylated or nonmethylated, suggesting that the methylation pattern was consistent across all retinal cell types (Fig. 6B).

DISCUSSION

Cell Death Pathways in Glaucoma

Our high-throughput comparative data obtained by quantitative LC-MS/MS analysis of retinal protein samples, along with the findings of quantitative Western blot analysis and tissue immunolabeling, reflected a prominent upregulation of apop-

FIGURE 2. Caspase activation in the glaucomatous human retina. Western blot analysis of retinal protein samples using cleavage site-specific antibodies detected cleaved caspases only in glaucomatous samples. Double immunofluorescence labeling indicated prominent localization of cleaved caspases 3, 8, 9, and 12 (green) in Brn-3-positive RGCs (red) in the glaucomatous human retina, whereas cleaved caspase 1 immunolabeling (green) was more prominent in Brn-3-negative cells corresponding to glia in the inner retina. rgc, retinal ganglion cell layer. Scale bar, 50 μ m.

tosis-related pathways and markers of inflammation in human glaucoma with many links to TNF- α /TNFR1 signaling.

We used a label-free approach for quantitative analysis of protein expression by LC-MS/MS. Because of the substantial cost of stable-isotope labeling along with the complexity of

sample processing and data interpretation, label-free methods have become more widely used. Although the quantitative assessment of spectral counts presents advantages, a potential caveat is the individual analysis of samples as opposed to mixing the comparative samples before analysis, which is a

FIGURE 3. NF- κ B activation in the glaucomatous human retina. Quantitative Western blot analysis used phosphorylation site-specific antibodies to validate NF- κ B activation. Based on β -actin-normalized intensity values, glaucomatous samples exhibited a prominent increase in phosphorylated p50 and p65 compared with nonglaucomatous control samples (Mann-Whitney rank sum test; $P < 0.01$). Double-immunofluorescence labeling of the glaucomatous human retina indicated localization of these active subunits (green) mainly in GFAP-positive astrocytes (red) in the inner retina. However, some GFAP-negative cells likely corresponding to RGCs (arrow) also exhibited weaker immunolabeling for these phospho-proteins. rgc, retinal ganglion cell; in, inner nuclear layer. Scale bar, 50 μ m.

FIGURE 4. STAT activation in the glaucomatous human retina. Quantitative Western blot analysis using phosphorylation site-specific antibodies and β -actin-normalized intensity values demonstrated increased STAT phosphorylation in glaucomatous samples compared with nonglaucomatous control samples. (Mann-Whitney rank sum test; $P < 0.01$). Double-immunofluorescence labeling indicated localization of these phosphorylated proteins (green) in GFAP-positive astrocytes (red) and also some GFAP-negative cells, likely corresponding to RGCs (arrow) in the inner retina. rgc, retinal ganglion cell; in, inner nuclear layer. Scale bar, 50 μ m.

primary advantage of labeling to eliminate experimental variability. However, previous studies^{4,5,7} using the label-free approach demonstrated a high level of reproducibility validated by immunoblotting and immunohistochemistry. Our findings here similarly support the usefulness of this method to identify valid expression changes even in relatively low-abundant proteins in glaucomatous tissues. Similar to LC-MS/MS, Western blot analysis-based quantification may be challenging primarily because of variability in transfer and amplification steps. With regard for the preparation-related variability in sample conditions, transfer efficiency, and backgrounds, we reprobated immunoblots with a β -actin antibody and repeated the analysis at least three times by running glaucomatous or nonglaucomatous samples on the same gel and in different combinations with similar results.

Retinal proteins exhibiting increased expression in human glaucoma included TNF- α , TNFR1, and various downstream adaptor/interacting proteins and protein kinases known to regulate diverse consequences of TNF- α /TNFR1 signaling. For example, a proteolytic caspase cascade leads to apoptosis after TNFR1 binding; however, the signaling cascade activating NF- κ B primarily promotes cell survival and regulates the expression of a wide variety of proteins that control innate and adaptive immunity.¹³ One of the specific molecules we detected was TRADD. This signal transducer protein is a component of the multiprotein signaling complex formed after TNFR1 binding, which recruits various proteins including members of the TRAF family. A number of proteins associated with the death receptor-mediated caspase cascade and NF- κ B activation appear to bifurcate at TRADD.¹⁴

In addition to data supporting the receptor-mediated caspase cascade, cell death signaling in the glaucomatous human retina exhibited links to the mitochondrial pathway.¹ Among various proapoptotic members of the Bcl-2 family regulating this pathway, we detected the upregulation of Bax, which is a principal regulator of RGC death.¹⁵ We also de-

tected (BH3)-only proapoptotic members of the family in glaucomatous samples, including Bid and Bim. With particular relevance to TNFR signaling, Bid participates in the activation of the mitochondrial cell death pathway on cleavage by caspase 8, a proximal caspase activated after TNFR1 binding.¹⁶ Previous studies have implicated Bid in RGC apoptosis in experimental glaucoma¹⁷ and Bim in RGC death after optic nerve axotomy.^{18,19} It has become clear that even with the lack of detectable change in their expression in animal models of glaucoma, (BH3)-only proteins potentiate Bax-mediated cell death by neutralizing antiapoptotic proteins such as Bcl-XL.²⁰

We also detected the increased expression of various ER-resident proteins, including stress-regulated chaperones that catalyze protein folding and function as sensors detecting unfolded protein response (UPR).²¹ Although UPR is an adaptive response to preserve cell function and survival, its persistence initiates apoptotic cascades, and has been implicated in the pathogenesis of multiple human diseases as in experimental glaucoma.²² In addition to UPR, disturbances in ER calcium homeostasis and redox changes may have important links to ER stress and communications with mitochondria.²³ By providing a unique oxidizing environment for disulfide bond formation during protein folding, ER may significantly contribute to mitochondria-generated oxidative stress.^{24,25} There appears to be a vicious relationship between ER stress and oxidative stress that is likely to play a role in increasing cellular susceptibility to neurodegenerative injury in glaucoma.

Our data also supported the increased expression of calpains in the glaucomatous human retina, which have been shown to contribute to neuronal death in ocular hypertensive rats.²⁶ Besides caspase-independent proteolytic activities, calpains cleave and activate an ER protein, caspase 12, thereby providing a link to the caspase-mediated apoptosis pathway.²⁷

Thus, our data support the coactivation of different apoptotic pathways in the glaucomatous human retina, including caspase- and calpain-mediated pathways, mitochondrial dys-

FIGURE 5. TNFAIP3 expression in the glaucomatous human retina. Quantitative Western blot analysis using a specific antibody revealed prominent individual differences in the expression level of TNFAIP3. When the average β -actin-normalized intensity value obtained from control samples was used to calculate the fold change in TNFAIP3 immunolabeling, we detected a greater than two-fold increased expression in 4 of 10 glaucomatous samples. * $P < 0.01$, statistically significant difference (Mann-Whitney rank sum test). However, TNFAIP3 expression was unchanged or decreased in the rest of the glaucomatous samples. Immunolabeling of human tissues demonstrated TNFAIP3 (green) localization in both Brn-3-positive RGCs and GFAP-positive astrocytes (red) in the inner retina. rgc, retinal ganglion cell; in, inner nuclear layer. Scale bar, 50 μ m.

function, and ER stress. Cross-talk between these pathways may reinforce each other during the apoptotic process in human glaucoma.

Immune Response Pathways in Glaucoma

Opposing the mediation of cell death, we also detected NF- κ B activation in the glaucomatous human retina, which plays an essential role as a key regulator of neuronal survival programs induced by TNF- α signaling.²⁸ NF- κ B may promote cell survival by inhibiting JNK and inducing antiapoptotic Bcl-2 members, IAPs, and HSPs.^{29,30} In addition, by controlling the transcription of immune mediators, NF- κ B regulates various aspects of

innate and adaptive immune responses.³¹ In glaucomatous samples, we detected the phosphorylation of NF- κ B1-p105/p50 and p65 (RelA), two of five distinct but structurally related subunits with specific signaling functions (the others are NF- κ B2-p100/52, RelB, and c-Rel). We also detected the increased expression of related kinases, such as RIPK, NIK, and I κ K, including a master regulator, I κ K γ (NF- κ B essential modulator [NEMO]). The activation of NF- κ B primarily occurs through activation of the I κ K, composed of a heterodimer of the catalytic I κ K α and I κ K β subunits and NEMO. Both p50 and p52 subunits participate in target gene transactivation by forming heterodimers with RelA, RelB, or c-Rel. In contrast to the

FIGURE 6. Cytosine nucleotide methylation differences in the *TNFAIP3* promoter among glaucomatous human donors. **(A)** Schematic representation of human *TNFAIP3* promoter methylation and location of the detected methylation sites. In glaucomatous retinas with low *TNFAIP3* expression (corresponding to glaucomatous donors 4, 5, and 9 in Fig. 5), three CpG and ten non-CpG methylation sites were found. However, in glaucomatous retinas exhibiting high expression of *TNFAIP3* (corresponding to glaucomatous donors 1 and 7 in Fig. 5), these sites remained unmethylated. *Circles*: non-CpG methylation sites; *triangles*: CpG methylation sites; *filled symbols*: methylated sites. **(B)** Examples of sequencing results of the *TNFAIP3* promoter after bisulfite conversion in donors exhibiting low (*top*) or high (*bottom*) level of *TNFAIP3* expression. In donors with high *TNFAIP3* expression, the conversion of cytosine residues was complete, suggesting that the vast majority of these residues were unprotected.

canonical signaling that relies on NEMO-I κ K-mediated degradation of I κ B followed by RelA/p50 signaling, noncanonical signaling critically depends on the NIK-mediated processing of p100 into p52 and RelB/p52 signaling.^{13,32} Diverse functions of NF- κ B as a master regulator of inflammatory responses and secondary injury processes in the CNS may depend on cell-specific factors and unbalanced activation of different subunit complexes.³³

In addition to various other kinase pathways, our proteomic data indicated JAK/STAT signaling in the glaucomatous human retina. This signaling pathway can be triggered in response to multiple stimuli, including TNFR1 binding, and mediates cytokine-mediated inflammatory responses in the CNS.^{34,35} Recent studies have documented that various components of the JAK/STAT signaling pathway are upregulated in the retina³⁶ and optic nerve³⁷ of ocular hypertensive rats.

Besides the caspases leading to apoptotic cell death, we detected caspase 1 activation in our glaucomatous samples. Stress-induced activation of caspase 1 is recognized as an essential regulator of inflammatory responses through its capacity to process and activate proinflammatory cytokines; therefore, this caspase is considered an inflammatory caspase. In addition to previous evidence supporting TLR signaling⁵ and complement activation,⁴ our new data support inflammasome assembly leading to caspase 1 activation in the glaucomatous

human retina. These cytosolic multiprotein complexes are assembled as an early innate response to cell stress, and activated caspase 1 initiates an inflammatory cascade by promoting the proteolytic activation of pro-interleukins and the secretion of mature cytokines.^{38,39} TNF- α signaling has been shown to promote inflammasome activation mediating sterile inflammation,^{40,41} and the NLRP3 inflammasome has been implicated in the development of neuroinflammation in the CNS.^{42,43}

Thus, presented data support that in addition to NF- κ B-mediated inflammation signaling, JAK/STAT signaling and inflammasome may be involved in immune response pathways activated in the glaucomatous human retina and may represent promising targets for immunomodulatory treatment strategies.

Regulation of TNF- α Signaling in Glaucoma

Our proteomic data indicated some specific regulator molecules. One of these molecules was CFLAR, a protease-deficient caspase homolog protein widely regarded as an apoptosis inhibitor.^{44,45}

We also detected optineurin in the human retinal proteome. Based on gene mutations detected among glaucoma patients, optineurin is proposed to be associated with TNF- α -mediated RGC death.⁴⁶ This TNF- α -inducible protein expressed by RGCs⁴⁷ appears to constitute a cellular stress sensor mecha-

nism transmitting survival signals.⁴⁸ A more recent study using microRNA silencing has shown that optineurin inhibits TNF- α -induced NF- κ B activation by competitively antagonizing NEMO for RIPK binding.⁴⁹ It is tempting to further determine whether gene mutations may affect the physiologic function of optineurin to dampen TNF- α signaling, thereby increasing neuronal susceptibility to glaucomatous injury.

Another important regulator molecule we detected was TNFAIP3; however, as verified by quantitative Western blot analysis, its expression level exhibited a prominent variability among glaucomatous donors. This cytoplasmic ubiquitin-editing zinc finger protein plays a key role in the negative regulation of TNF- α signaling by functioning as a dual inhibitor of NF- κ B activation and TNF- α -mediated apoptosis.⁵⁰ By antagonizing interactions with ubiquitin-conjugating enzymes, TNFAIP3 may inactivate various molecules downstream of TNFR1,⁵¹ block JNK activation, and inhibit proteolytic cleavage of caspase 8.^{52,53} TNFAIP3-mediated inhibition of the caspase cascade effectively protects neurons from postischemic apoptosis in the CNS.⁵⁴ In addition to antiapoptotic activities, TNFAIP3 is involved in the negative feedback regulation of NF- κ B signaling by its interaction with various upstream signaling molecules, modulating their ubiquitination and proteasome-mediated degradation.^{55,56} TNFAIP3, acting through NF- κ B signaling, restricts innate and adaptive immune responses and ensures the transient nature of inflammatory signaling. Consequently, reduced TNFAIP3 expression is suggested to predispose to autoimmunity as well as increasing the susceptibility to neuronal injury.^{50,57} The essential role of TNFAIP3 in the regulation of apoptosis and NF- κ B signaling has been clearly demonstrated with the generation of TNFAIP3 knockout mice, which develop severe inflammation in multiple organs and die prematurely at 3 to 6 weeks of age. TNFAIP3-deficient cells fail to terminate TNF- α -induced NF- κ B activation and become more susceptible to TNF- α -mediated apoptosis.⁵⁸ In addition, the RNA interference-mediated downregulation of TNFAIP3 in human dendritic cells results in enhanced stimulation of cytotoxic T cells and inhibition of regulatory T cells.^{59,60} Given the key functions of TNFAIP3 in the regulation of cell death and the prevention of autoimmunity, it would be interesting to determine whether aberrations in its expression may increase RGC susceptibility to TNF- α -mediated apoptosis or may alter the intensity or duration of immune responses in glaucoma.

Consistent with previous experimental findings, recent genetic studies have demonstrated several mutations in the human *TNFAIP3* locus as risk alleles for multiple autoimmune diseases in humans.⁵⁰ Findings of these studies motivated us to determine whether the variability in TNFAIP3 expression among glaucomatous donors reflects a similar association. We therefore initiated analyses of genetic and epigenetic differences across these samples. Despite the lack of any detectable genomic variation correlated to individual differences in protein expression, our data obtained from bisulfate sequencing demonstrated that the methylation of cytosine nucleotides in the *TNFAIP3* promoter is correlated with the variability in retinal protein expression among glaucomatous donors. Although bisulfate sequencing is inherently challenging, as the electropherograms demonstrated, potential problems such as bisulfate treatment-related DNA degradation, incomplete conversion, or differential PCR amplification rates of converted and unconverted sequences did not occur in our hands. Cytosine nucleotide methylation is one of the most critical epigenetic mechanisms for gene silencing described for *TNFAIP3*. This gene has been shown to be inactivated because of partial methylation of several CpG sites upstream of exon 1.⁶¹ In the DNA extracted from glaucomatous retinas, we observed methylation of only one of these sites but detected the methylation

of several cytosine residues not followed by guanine residues. Although cytosine methylation of the CpG dinucleotide is well documented,⁶² non-CpG methylation has more recently been described,^{63,64} and emerging data indicate that this type of methylation may result from de novo methylation mediated by the methyltransferases DNMT3a and DNMT3b.^{65,66} It is of interest to note that differential methylation may also be related to differential demethylation caused by demethylase,⁶⁷ glycosylase,⁶⁸ or other related enzymatic activities.⁶⁹ It appeals further studies to determine whether detected methylation differences in the *TNFAIP3* promoter may represent an epigenetic modification that could modify a person's response to TNF- α -mediated processes in glaucoma.

Diverse Consequences of TNF- α Signaling in Glaucoma

By highlighting various proteins linked to TNF- α /TNFR1 signaling in the glaucomatous human retina, findings of this study support that a complex cross-talk relationship between multiple signaling pathways determines diverse consequences of TNF- α signaling.³ Factors determining opposing effects of TNF- α signaling also include the type of receptor preferentially used. Two cell surface receptors, p55 (TNFR1) and p75 (TNFR2), mediate biological activities of TNF- α . These two receptors are co-expressed on most cell types and feed into diverse signaling pathways according to differences in their intracellular domains. A death domain in TNFR1, not present in TNFR2, leads to apoptotic cell death, whereas signaling through TNFR2 leads primarily to cell proliferation. Similarly, TNFR1 has been found to augment neuronal death and TNFR2 has been found to promote neuroprotection in a retinal ischemia model in knockout mice.⁷⁰ No increase was detectable in the expression of TNFR2 in the glaucomatous human retina. A recent study⁷¹ of an experimental rat glaucoma model has supported that signaling through TNFR2 may be neurotoxic through a paracrine mechanism by increasing the glial production of neurotoxic proteins, including TNF- α . Another study⁷² has similarly shown that activation of this receptor may trigger RGC death through a non-cell-autonomous signaling pathway by inducing TNF- α production in Müller cells. These findings collectively suggest that our proteomic data supportive of TNF- α -mediated cell death signaling in human glaucoma may predominantly reflect TNFR1 signaling. Regarding inflammation signaling, studies using receptor-specific antibodies,⁷³ ligands,⁷⁴ and knockout mice⁷⁵⁻⁷⁷ have indicated that TNFR1 is the primary signaling receptor on most cell types through which the majority of inflammatory responses classically attributed to TNF- α occur. Furthermore, soluble TNF- α , more than its membrane-bound form, is required to generate neuroinflammation,⁷⁸ which is the principal ligand for TNFR1.⁷⁹ Thus, TNFR1 appears to be the primary receptor for both neurodegenerative and inflammatory consequences of TNF- α signaling in glaucoma. However, any contribution of TNFR2 signaling to retinal proteomic components in glaucoma must be further determined by loss-of-function studies.

Diverse functional characteristics of TNF- α may also be attributed to time-dependent factors and the duration of TNF- α signaling. Because activated glial cells are the major source of increased TNF- α production in glaucoma, the activation status of different glial cell types and the time course of specific glial responses are particularly important. Our parallel studies using a cell-specific proteomic approach in animal models should facilitate improved understanding of the receptor type-related, cell type-specific, and time-dependent components of TNF- α signaling and should help identify new treatment targets for glaucoma.

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