An Astrocyte-Specific Proteomic Approach to Inflammatory Responses in Experimental Rat Glaucoma

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PURPOSE. To delineate astrocyte-mediated inflammatory processes in glaucoma, we analyzed proteomic responses of retinal astrocytes in an experimental rat model using a cell-specific approach.

METHODS. IOP elevation was induced in rats by hypertonic saline injections into episcleral veins. Enriched samples of astrocytes were isolated through the immunomagnetic cell selection process established originally for retinal ganglion cell (RGC) sampling. Ocular hypertensive and control samples were collected by pooling from rat eyes matched for the cumulative IOP exposure. Protein expression was analyzed complementarily by quantitative two-dimensional capillary liquid chromatography and linear ion trap mass spectrometry (LC-MS/MS) followed by quantitative Western blot analysis and retinal tissue immunolabeling using specific antibodies to selected proteins.

RESULTS. Following validation of enriched astrocyte samples, LC-MS/MS analysis resulted in the identification of over 2000 proteins with high confidence. Bioinformatic comparison analysis of the high-throughput MS/MS data along with the findings of immunoblotting and immunohistochemistry supported distinct responses of ocular hypertensive astrocytes during the experimental paradigm, which exhibited predominantly cellular activation and immune/inflammatory responses as opposed to activation of cell death signaling in ocular hypertensive RGCs. Inflammatory responses of astrocytes in experimental glaucoma included up-regulation of a number of immune mediators/regulators linked to TNF-α/TNFR signaling, nuclear factor kappa-B (NF-κB) activation, autophagy regulation, and inflammasome assembly.

CONCLUSIONS. These findings validate an astrocyte-specific approach to quantitatively identify proteomic alterations in experimental glaucoma, and highlight many immune mediators/regulators characteristic of the inflammatory responses of ocular hypertensive astrocytes. By dissecting the complexity of prior data obtained from whole tissue, this pioneering approach should enable astrocyte responses to be defined and new treatments targeting astrocytes to be developed. (*Invest Ophthalmol Vis Sci.* 2012;53:4220–4233) DOI: 10.1167/iovs.11-9101

Retinal ganglion cell (RGC) axons, somas, and synapses are specific victims of glaucomatous neurodegeneration, but glial cells, including retina and optic nerve astrocytes, survive the glaucomatous tissue stress and respond differently. By exerting both neurosupportive and detrimental effects, glial cells have key roles in determining neuronal life or death decisions in glaucoma. It has become clear over the past two decades that elucidation of RGC and glia responses are equally important for glaucoma research aiming to better understand and treat neurodegeneration.[1]

An unbalanced environment created by a variety of stress stimuli in glaucomatous tissues becomes a major initiator and propagator of secondary injury processes, which include neuroinflammation.[1,2] Chronic activation of the glia, resident immune regulatory cells, is commonly accepted as an indicator of ongoing neuroinflammation in the glaucomatous retina and optic nerve.[1] A growing number of studies analyzing gene and protein expression in these tissues support increased production of various immune mediators in human glaucoma[3–5] and different animal models.[6–11] Based on in vitro observations, glial immune mediators are important to establish autocrine and paracrine feedback circuits for innate immune injury, glia-T cell interactions, and antigen presentation.[12] For example, TNF-α, which is a major pro-inflammatory cytokine produced increasingly by activated glial cells in glaucoma,[13,14] has been linked to glial activation response, inflammatory processes, and mediation of RGC death in cell cultures.[15–17]

We previously used enriched samples of RGCs in proteomic analysis to illuminate different aspects of RGC responses during glaucomatous neurodegeneration.[18–20] More recently, we also started to isolate enriched samples of astrocytes through a similar cell isolation technique. With the advantage of cell-specific sampling, our study aimed to determine astrocyte-mediated inflammatory processes in an experimental rat model of glaucoma. Our findings highlighted various molecules characteristic of the distinct inflammatory responses of astrocytes during the experimental paradigm. Dissection of cell-specific responses can help identify molecular pathways of glaucomatous neurodegeneration toward new treatment strategies, and better understanding of glial immune response pathways can lead us to immune modulatory treatments for neuroprotection.

MATERIALS AND METHODS

Experimental Rat Model of Glaucoma

Similar to previous studies,[19–21] IOP elevation was induced in 8-month-old Brown Norway rats by hypertonic saline injections into episcleral

veins as originally described by Morrison et al.[22] IOP was measured in awake rats twice weekly using a handheld rebound tonometer (TonoLab, Colonial Medical Supply, Franconia, NH) and monitored for up to 8 weeks.

To determine optic nerve injury, 1 μm plastic cross-sections of the optic nerves were used for imaging-based axon quantification as described in our previous studies.[19-21] However, unlike previously used systematic sampling protocol, optic nerve cross-sections were imaged in their entirety as non-overlapping frames using the Zeiss/AxioVision/MosaiX-Panorama software (Carl Zeiss, Thornwood, NY). This methodological improvement allowed axon counts representing the entire surface area of optic nerve cross-sections, free from sampling bias. After image acquisition, processing and analysis of captured images were performed as described previously using the Axiovision software (Carl Zeiss).[19-21] By following the same protocol, we manually traced nerve outlines on mosaics of images, and determined the size and shape parameters to exclude intervening glia, myelin debris, and highly degenerated axons to ensure accurate counts.

Cumulative IOP exposure was determined by calculating the area under the pressure-time curve in the ocular hypertensive eye, then subtracting this IOP-time integral from that in the normotensive fellow eye (expressed in units of mm Hg-days) as described previously.[21] To minimize the influence of IOP variability among animals and within the same eye over time, cell-specific samples of retinal proteins were collected by pooling from rat eyes matched for the cumulative IOP exposure of 200–400 mm Hg-days. Based on optic nerve axon counts, this selection criterion corresponded to a relative axon loss value of no more than 50% (42.6 ± 7.3%). Animals with no measurable IOP exposure were excluded from further analysis.

Mass spectrometric analysis and following Western blot analysis for data validation required cell-specific protein samples collected from over 200 rats. In an adult rat retina approximately 150,000 RGCs are present and the rat retina exhibits an astrocyte-to-RGC ratio of 1:5–7. The cell selection procedure used (with cell yields of more than 75%) isolates at least 50 μg of astrocyte protein from 15 rat eyes with or without ocular hypertension. Although 50 μg or more RGC protein can be obtained by pooling from 2–3 normotensive rat eyes, the same amount of RGC protein can be obtained from 8–9 ocular hypertensive rat eyes with 50% axon loss.

All animals were handled according to the regulations of the Institutional Animal Care and Use Committee, and all procedures adhered to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Sampling of Enriched Astrocyte Proteins

Enriched samples of astrocytes were obtained through the immunomagnetic technique we described originally to isolate RGCs.[15] Briefly, retinas were dissected from enucleated rat eyes and the neural retina was peeled gently from the retinal pigment epithelium under a microscope. Tissues were dissociated briefly in Eagles's minimum essential medium (EMEM) containing 20 U/mL papain, 1 mM L-cystein, 0.5 mM EDTA, and 0.005% DNase (Worthington, Lakewood, NJ). After rinsing in an inhibitor solution containing EMEM, 0.2% ovomucoid (US Biological, Swampscott, MA), 0.04% DNAase, and 0.1% BSA (Sigma-Aldrich, St. Louis, MO), gentle trituration through a 1 mL plastic pipette was used to yield a suspension of single cells.

The immunomagnetic technique used antibody coated magnetic beads (4×10^8 beads/ml; Invitrogen, Grand Island, NY). After blocking by incubation with 1% gamma globulin, retinal cells were incubated with a monoclonal ASTRO1 antibody specific to an astrocyte surface protein for 20 minutes (100 μg for 1×10^5 cells/ml; Abcam, Cambridge, MA). Incubation was followed by washing steps with PBS containing 0.1% BSA, and 2 mM EDTA, pH 7.4. To select astrocytes, cells then were incubated with anti-mouse IgM-coated magnetic beads (25 μl; Invitrogen) for 20 minutes at 4°C. For complementary comparative analysis, we also obtained enriched samples of RGCs using a monoclonal antibody to Thy-1.1 (100 μg/ml; Millipore/

Chemicon, Billerica, MA) as described previously.[15,18-20] Protein samples were prepared with a lysis buffer containing 50 mM Hepes-KOH at pH 8.0, 100 mM KCl, 2 mM EDTA, 0.10% NP-40, 2 mM dithiothreitol, 10% glycerol, and protease and phosphatase inhibitors as described previously.[23]

All quantitative analyses of protein expression were repeated at least three times with different sample pools. The Mann-Whitney rank sum test was used to determine the statistical significance of differences in protein expression between ocular hypertensive and control samples.

Proteomic Analysis

Cell-specific samples (10 μg) were analyzed quantitatively by two-dimensional capillary liquid chromatography and linear ion trap mass spectrometry (LC-MS/MS) as described previously.[23] Briefly, trypsin-digested samples were loaded onto an analytical 2D-capillary chromatography column packed with strong cation exchange (SCX) and C_{18} 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 500 mM ammonium acetate and eluted into a linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) according to a linear HPLC gradient. Protein identification was performed with Sequest Sorcerer (Sage-N Research, San Jose, CA) set up to search a FASTA formatted rat protein database with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.2 Da. The Scaffold (Proteome Software Inc., Portland, OR) was used to validate peptide and protein identifications based on the criteria of greater than 95.0% and 99.0% probability, and at least two peptides as specified by the Prophet peptide[24] and protein[25] algorithms. The protein abundance was determined by normalizing the number of unique spectral counts matching to the identified protein by its predicted molecular weight.[26]

Similar to previous studies,[3-5] we used the Ingenuity Pathways Analysis (Ingenuity Systems, Mountain View, CA) to define functional patterns and generate interaction networks of the identified proteins. Canonical pathway analysis identified the pathways from the Ingenuity Pathways Analysis library that were most significant to the data set by the right-tailed Fisher's exact test.

Quantitative Western Blot Analysis

For proteomic data validation, cell-specific samples (10 μg) were also studied by quantitative Western blot analysis as described previously.[3-5] Briefly, SDS-PAGE used 7.5% or 12% gels (Bio-Rad, Hercules, CA), and separated proteins were transferred electrophoretically to a nitrocellulose membrane (Bio-Rad). Following a blocking step, membranes were probed with a primary antibody to ASTRO1 (1:100; Abcam), glial fibrillary acidic protein (GFAP; 1:1000; Abcam), Thy-1.1 (1:1000; Millipore/Chemicon), neuronal nucleus protein (NeuN; 1:1000; Millipore/Chemicon), glutamine synthetase (GS; 1:1000; Millipore/Chemicon), cellular retinaldehyde-binding protein (CRALBP; 1:2000; Abcam), retinal pigment epithelium-specific protein 65 (RPE65; 1:5000; Abcam), TNF-α (1:500; Abcam), TNF receptor-1 (TNFR1; 1:500; Abcam), cleaved caspase-8 (1:500; Cell Signaling, Danvers, MA), a subunit of nuclear factor-kappa B (NF-κB), p65 (phospho-Ser276, 1:1000; Cell Signaling), an immunity-related GTPase (IRG), IRGM (1:500; LifeSpan Biosciences, Seattle, WA), mammalian target of rapamycin (mTOR; phospho-Ser2448, 1:100; Cell Signaling), nucleotide-binding domain, leucine-rich repeat containing (NLR) family-pyrin domain containing 3 (NLRP3; 1:500; Abcam), or procaspase-1/cleaved caspase-1 (1:150; Millipore/Chemicon). The antibody dilutions used were optimum as assessed preliminarily by signal intensity, background staining, and amount of non-specific detection with varying antibody concentrations. After a second blocking step, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (1:2000; Sigma-Aldrich). Immunoreactive bands were visualized by enhanced chemi-

luminescence using commercial reagents (GE Healthcare, Pittsburgh, PA). Images were captured at optimum condition for proper balance between signal and background, and avoided band saturation with signal. A beta-actin antibody (1:1000; Sigma-Aldrich) was used to reprobe the stripped immunoblots for loading and transfer control.

For densitometry of immunoblots, images were acquired using a scanning device with a linear dynamic range (Typhoon 9400, GE Healthcare), image analysis used the ImageQuant software (GE Healthcare), background correction was applied to each lane by baseline subtraction, and integral values were selected as measurement parameters. Following normalization to beta-actin, the average values obtained from normotensive control samples was used to calculate the fold change in protein expression in ocular hypertensive samples.

Retinal Immunolabeling

To determine the cellular localization of selected proteins, histological sections of the rat retina were analyzed by immunohistochemistry as described previously.[3,4] Immunofluorescence labeling used the same primary antibodies described above for Western blot analysis, including antibodies to TNF-α, TNFR1, cleaved caspase-8, phospho-p65, IRGM, phospho-mTOR, NLRP3, and procaspase-1/cleaved caspase-1. In addition, a specific antibody against GFAP (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) was used to identify astrocytes. A mixture of Alexa Fluor 488- or 568-conjugated species-specific IgGs (1:500, Invitrogen) was used for the secondary antibody incubation. Similar to Western blot analysis, optimum antibody dilutions (1:100–500) were pre-selected based on retinal immunolabeling with minimum non-specific background staining. DAPI (Thermo Fisher Scientific) was used for nuclear counterstaining. Slides were examined by fluorescence microscopy and images were recorded by digital photomicrography (Carl Zeiss). Negative controls were performed by replacing the primary antibody with serum or using an inappropriate secondary antibody to determine species specificity. At least six histological sections obtained from three ocular hypertensive and control retinas were used for each antibody immunolabeling.

RESULTS

Our data validated the feasibility of astrocyte-specific sampling to detect cell-specific differences in protein expression in experimental glaucoma. We initially aimed to verify our enriched astrocyte samples by Western blot analysis using specific antibodies to different retinal cell markers. Similarly isolated RGC samples were verified previously based on retrograde fluorescence labeling,[15] cell morphology and immunolabeling in culture,[15] RT-PCR,[20] and Western blot analysis of specific markers.[18] However, to provide complementary information, we re-examined RGC samples, and also retinal protein samples obtained after depletion of astrocytes and RGCs, along with the analysis of astrocyte samples (Fig. 1A). Western blots verified GFAP and ASTRO1 expression in selected astrocytes. However, neuronal markers, including NeuN and Thy-1.1, were not detectable in these samples. Despite faint immunoreactivity for GS (a marker for Müller cells) and CRALBP (expressed in Müller cells and retinal pigment epithelium), enriched samples of astrocytes were negative for RPE65, a marker for retinal pigment epithelium. Similar to previous observations, RGC samples were positive for NeuN and Thy-1.1, but negative for ASTRO1, GFAP, GS, CRALBP, or RPE65. The minimum contamination of enriched astrocyte samples with Müller cell proteins (GS and CRALBP expressed only by developing astrocytes[27]) likely is due to the ASTRO1 antibody used for cell isolation, which is specific for astrocytes but also may bind weakly some Müller cells in the retina. To provide an internal control and complementary information, Figure 1A also presents Western blots of retinal protein samples obtained after depletion of astrocytes and RGCs. Note that dark immunoreactive bands for GS and CRALBP support Müller cell proteins remaining in these astrocyte/RGC-depleted samples. Altogether, the data presented in Figure 1A indicate no prominent contamination of enriched astrocyte samples with other retinal cell types and support the high purity of these samples.

The quantitative LC-MS/MS analysis of enriched astrocyte samples identified 2104 proteins by two peptides or more at the 0.2% peptide and 0.1% protein false discovery rates, and the MS/MS data included proteins exhibiting up-regulated or down-regulated expression in ocular hypertensive samples. Bioinformatic comparison analysis using the Ingenuity Pathways Analysis supported distinct responses of ocular hypertensive astrocytes during the experimental paradigm. To provide overall information about our high-throughput data, Figure 1B shows the functional groups of up-regulated proteins in ocular hypertensive astrocytes in comparison to ocular hypertensive RGCs. Ocular hypertensive astrocytes exhibited predominantly cellular activation and immune/inflammatory responses as opposed to the stress response and cell death signaling prominent in RGCs.

The Table lists 50 astrocyte proteins most relevant to inflammatory responses in ocular hypertensive samples. Data presented in this table include GFAP expression verifying astrocyte samples. Additional proteomic data are given in the Supplementary Table (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9101/-/DCSupplemental). Selected proteins from this list were studied further for data validation. We ran Western blot analysis to validate increased expression and/or activation of selected proteins. As an additional effort to provide further validation for distinct responses of astrocytes during glaucomatous neurodegeneration, we also analyzed enriched samples of RGCs for these proteins and presented our astrocyte data along with this complementary data from RGC samples. In addition, we immunolabeled histological sections of the rat retina with specific antibodies to verify the cellular localization of selected proteins in astrocytes.

As listed in the Table, identified immune mediators/regulators in ocular hypertensive astrocyte samples included a number of downstream proteins linked to TNF-α/TNFR signaling. As an initial step for data validation, we aimed to determine whether cell-specific samples reflect previously detected differences in TNF-α and TNFR1 expression in human glaucoma.[5,14] In parallel to previous observations, Western blot analysis supported increased expression of TNF-α and TNFR1 in astrocyte samples isolated from ocular hypertensive eyes relative to normotensive controls. This observation was also consistent with TNF-α immunolabeling localized predominantly to GFAP-positive astrocytes in the ocular hypertensive rat retina. However, TNFR1 immunolabeling was detectable on both GFAP-positive and GFAP-negative cells in the RGC/nerve fibers layers of the same tissues (Fig. 2).

The proteins linked to TNF-α/TNFR signaling in ocular hypertensive astrocytes mainly included mediators of inflammatory processes, not mediators of apoptosis. In contrast, inflammatory proteins were not detectable by mass spectrometric analysis of ocular hypertensive RGCs. Due to prominent up-regulation of TNFR signaling in ocular hypertensive samples, the next set of validation studies determined cell-specific differences in TNFR1-mediated caspase activation by Western blot analysis and immunohistochemistry using a cleavage site-specific antibody. In support of the MS/MS data, cleaved caspase-8 (a proximal caspase in the TNFR1-mediated apoptosis pathway) was detectable in ocular hypertensive RGCs, but not in astrocytes (Fig. 3).

While caspase activation was not detectable in ocular hypertensive astrocytes, the opposing pathway of TNFR
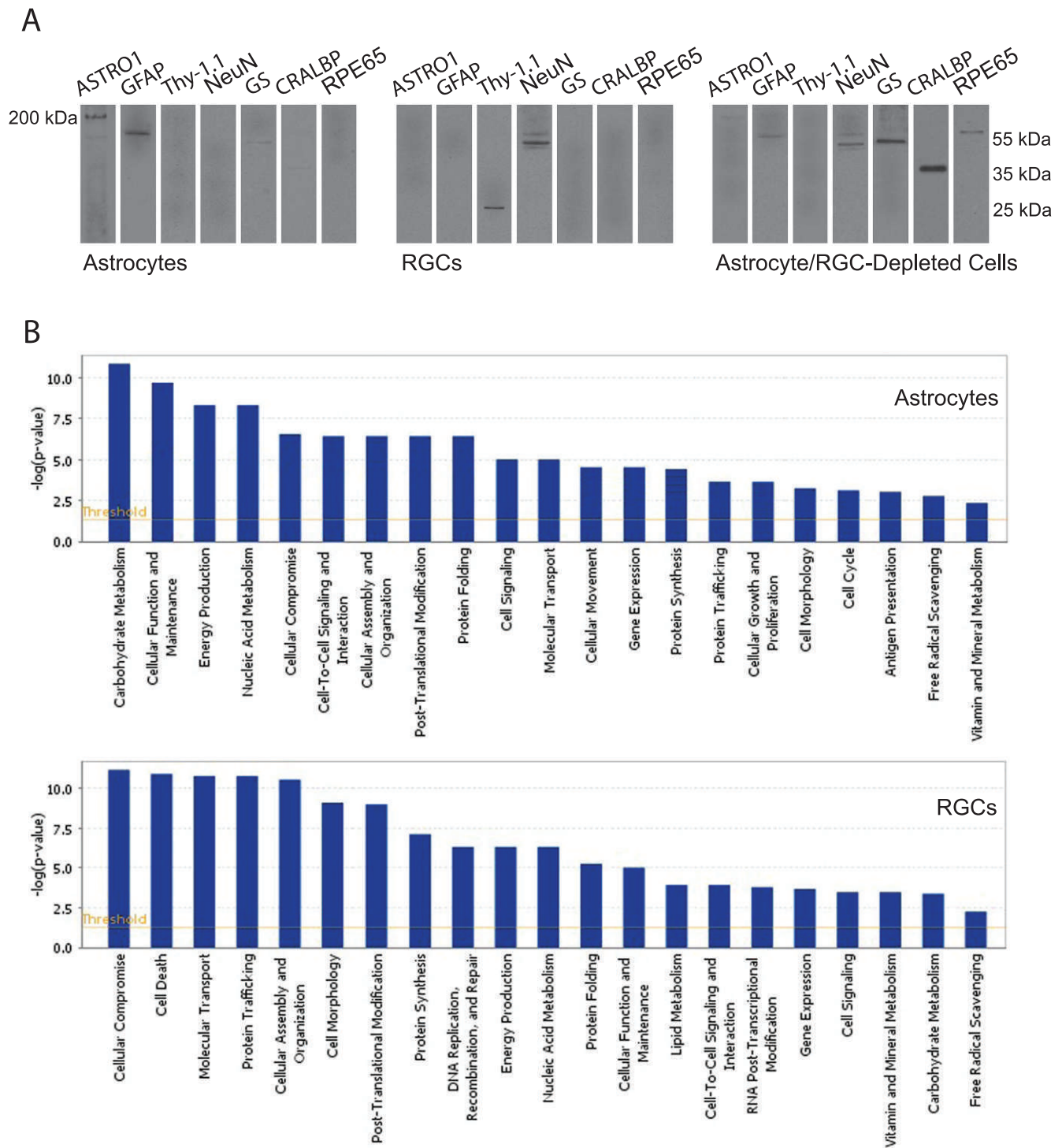
FIGURE 1. Validation of enriched astrocyte samples. (**A**) Enriched samples of astrocytes exhibited prominent immunoreactivity for ASTRO1 and GFAP. Despite faint immunoreactive bands for GS (a marker for Müller cells) and CRALBP (expressed in Müller cells and retinal pigment epithelium), enriched samples of astrocytes were negative for RPE65, a marker for retinal pigment epithelium, as well as for neuronal markers (including NeuN and Thy-1.1). However, Western blot analysis detected neuronal markers (NeuN and Thy-1.1) in RGC samples. Also presented are Western blots of retinal protein samples obtained after depletion of astrocytes and RGCs. Note the dark immunoreactive bands for GS and CRALBP in these astrocyte/RGC-depleted samples, which support remaining Müller cell proteins. Protein samples were separated using 12% SDS-PAGE gels (except for ASTRO1 immunoblots in which 7.5% gels were used), and membranes were cut into strips to probe with different antibodies specific to target proteins. Data represent three independent sets of analyses with different samples. (**B**) Bioinformatics analysis of the comparative proteomic data by the Ingenuity Pathways Analysis supported distinct responses of astrocytes and RGCs in ocular hypertensive eyes. Shown are functional groups of the up-regulated proteins in ocular hypertensive astrocytes and RGCs relative to their controls.

TABLE. Astrocyte Proteins Linked to Inflammatory Responses

Symbol	Protein Name	Protein ID	Fold Change
TRADD	TNFRSF1A-associated via death domain	gi|281427214	2.1*
PEA15	Astrocytic phosphoprotein PEA-15	gi|61557370	1.8*
TNFAIP2	Tumor necrosis factor alpha-induced protein 2	gi|212549631	1.9*
RelA	Nuclear factor NF-kappa-B subunit p65	gi|40538870	3.1*
NFKB-2	Nuclear factor NF-kappa-B subunit p100	gi|56605774	5.4*
IKBIP	Inhibitor of nuclear factor kappa-B kinase-interacting protein	gi|148539967	1.9*
MKK1	Dual specificity mitogen-activated protein kinase kinase 1	gi|13928886	2.2*
MKK3	Mitogen activated protein kinase kinase 3	gi|197333734	1.8*
MKK4	Dual specificity mitogen-activated protein kinase kinase 4	gi|71795640	1.5
ERK2	Mitogen-activated protein kinase 1	gi|16758698	1.2
ERK1	Mitogen-activated protein kinase 3	gi|8393331	3.2*
P38	Mitogen-activated protein kinase 14	gi|62461582	2.4*
ROCK2	Rho-associated protein kinase 2	gi|6981478	−1.4
AKT1	RAC-alpha serine/threonine-protein kinase 1	gi|15100164	2.8*
PDLIM1	PDZ and LIM domain protein 1	gi|8393153	1.1
PDLIM7	PDZ and LIM domain protein 7	gi|27465579	1.4
SGN1	COP9 signalosome complex subunit 1	gi|42476092	2.3*
SGN2	COP9 signalosome complex subunit 2	gi|23463271	−1.2
SGN3	COP9 signalosome complex subunit 3	gi|51948372	3.9*
SGN4	COP9 signalosome complex subunit 4	gi|51948518	0.2
SGN5	COP9 signalosome complex subunit 5	gi|71043620	1.6
SGN6	COP9 signalosome complex subunit 6	gi|157821399	1.2
COPS7A	COP9 complex subunit 7a isoform 2	gi|255760057	1.7
SGN8	COP9 signalosome complex subunit 8	gi|61557351	−0.7
ASCC311	Activating signal cointegrator 1 complex subunit 3-like	gi|281371480	4.1*
AP1B1	AP-1 complex subunit beta-1	gi|8392872	1.4
AP1G1	AP-1 complex subunit gamma-1	gi|189491695	1.2
AP1M1	AP-1 complex subunit mu-1	gi|112984344	2.2*
AP1S1	AP-1 complex subunit sigma-1	gi|205360945	−0.9
STAT1	Signal transducer and activator of transcription 1 isoform alpha	gi|77695926	2.5*
STAT2	Signal transducer and activator of transcription 2	gi|58865380	1.6
STAT3	Signal transducer and activator of transcription 3	gi|6981592	2.0*
STAT6	Signal transducer and transcription activator 6	gi|113205500	3.1*
TLR2	Toll-like receptor 2	gi|38454274	2.1*
TOLLIP	Toll-interacting protein	gi|157823763	−1.6
IRGQ	Immunity-related GTPase family, Q	gi|209364508	1.8*
mTOR	Serine/threonine-protein kinase mTOR	gi|9845251	2.1*
ATG3	Ubiquitin-like-conjugating enzyme autophagy-related 3	gi|19705511	1.5
ATG7	Ubiquitin-like modifier-activating enzyme autophagy-related 7	gi|58865764	2.7*
ASC	Apoptosis-associated speck-like protein containing a CARD	gi|27229294	1.9*
CASP1	Caspase-1	gi|31542341	2.0*
ILF2	Interleukin enhancer-binding factor 2	gi|114145744	0.5
ILF3	Interleukin enhancer-binding factor 3	gi|16758150	1.2
IL-1RA	Interleukin-1 receptor antagonist protein	gi|11559964	−1.9*
TGFB2	Transforming growth factor beta-2	gi|13592109	1.3
TGFB3	Transforming growth factor beta-3	gi|6981650	0.3
MIF	Macrophage migration inhibitory factor	gi|13591985	1.5
GMFB	Glia maturation factor beta	gi|13624295	1.8*
GMFG	Glia maturation factor gamma	gi|30842809	2.2*
GFAP	Glial fibrillary acidic protein	gi|158186732	1.6

Protein samples of enriched astrocytes were collected by pooling from rat eyes matched for the cumulative IOP exposure and analyzed by quantitative LC-MS/MS. All listed proteins were identified with high confidence (based on at least two peptides with greater than 99.0% probability).

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

signaling that promotes NF-κB activation was detectable prominently. Based on the Ingenuity Pathways Analysis, the NF-κB activation pathway was among the top canonical pathways associated most significantly with our astrocyte dataset (right-tailed Fisher's exact test, $P < 0.05$). Western blot analysis using a phosphorylation site-specific antibody indicated increased phosphorylation of p65 (RelA) in ocular hypertensive astrocytes. Based on immunohistochemical anal-

ysis, phospho-p65 was localized predominantly to GFAP-positive astrocytes in the ocular hypertensive rat retina (Fig. 3).

Another group of proteins up-regulated in ocular hypertensive astrocytes was related to autophagy signaling, including mTOR and autophagy-related proteins (ATGs), ATG3 and ATG7. Immunolabeling with a phosphorylation site-specific antibody also indicated phosphorylation-mediated activation of mTOR in ocular hypertensive astrocytes. However, phospho-mTOR was not prominently detectable in ocular hypertensive
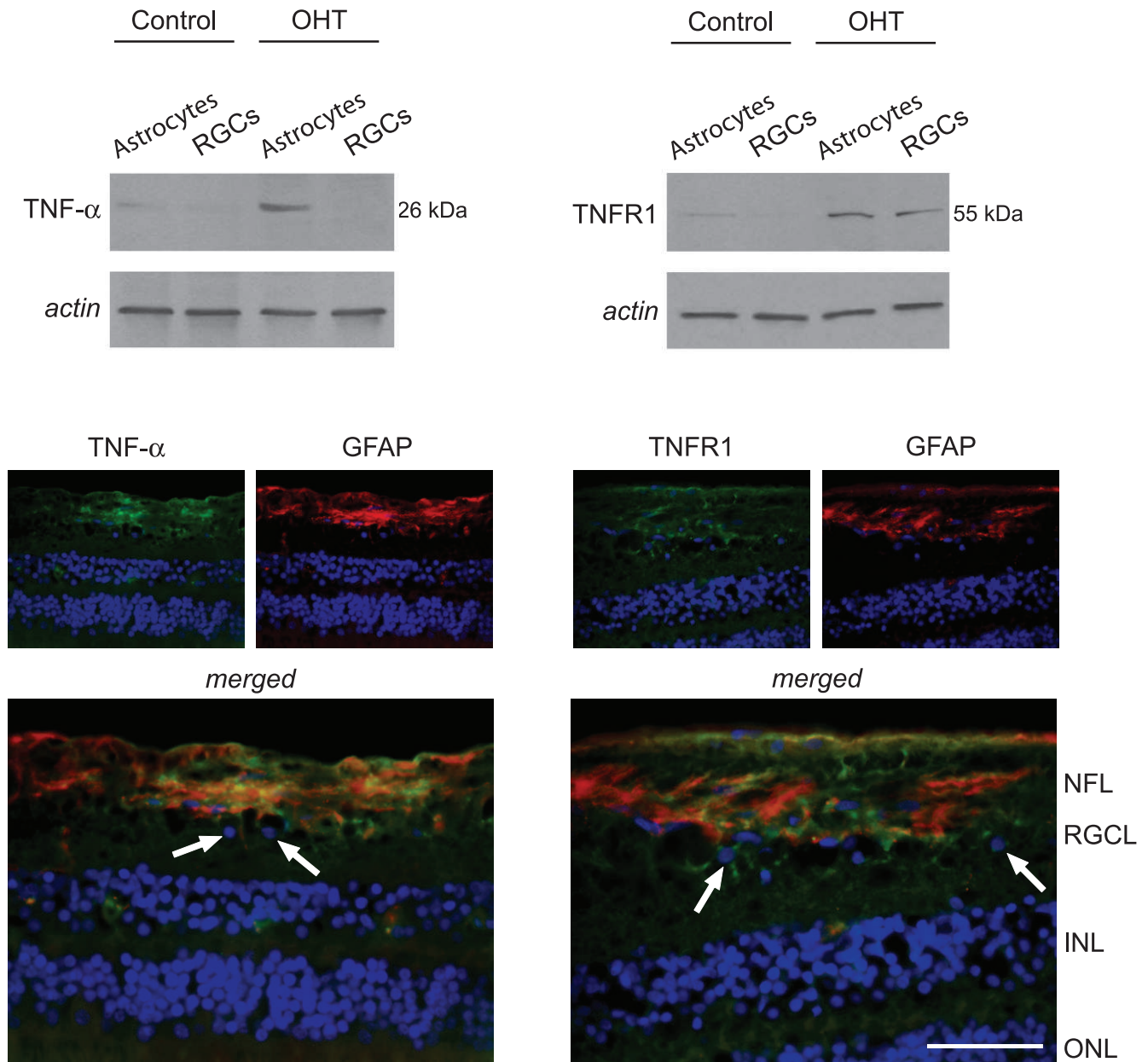
FIGURE 2. TNF-α and TNFR1 expression in astrocytes. Western blot analysis detected TNF-α expression in astrocyte samples, not in RGCs. However, TNFR1 expression was detectable in samples of RGCs and astrocytes isolated from ocular hypertensive (OHT) rat eyes. Immunohistochemistry images show the OHT retina that indicated prominent localization of TNF-α (*green*) in GFAP-positive (*red*) astroglia. GFAP-positive cells in the RGC and NFL correspond to astrocytes, while Müller cells (located in the inner nuclear layer) and their processes through the retina may also exhibit immunolabeling for GFAP in OHT animals similar to human glaucoma. Regarding TNFR1 immunolabeling (*green*), in addition to GFAP-positive astroglia, some GFAP-negative cells in the RGC layer, likely corresponding to RGCs (*arrows*), also exhibited TNFR1 immunolabeling in the OHT rat retina. *Blue* corresponds to nuclear DAPI labeling. Data represent three independent sets of analyses with different samples. NFL, nerve fibers layer; RGCL, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar 100 μm.

RGCs. An IRG linked to immunity-related autophagy, IRGM, also exhibited increased expression by Western blot analysis of ocular hypertensive astrocytes (Fig. 4).

Western blot analysis also validated NLRP3 expression and caspase-1 cleavage supporting inflammasome assembly in ocular hypertensive astrocytes. In addition, immunohistochemical analysis of ocular hypertensive rat retinas indicated prominent localization of these inflammasome components in GFAP-positive astrocytes (Fig. 5).

Figure 6 summarizes the protein expression data based on quantitative Western blot analysis. Figure 7 shows a simplified network of the studied proteins that characterize inflammatory responses of ocular hypertensive astrocytes.

DISCUSSION

Cell-Specific Approach to Glaucomatous Neurodegeneration

Our study used a cell-specific approach to determine retinal astrocyte responses in experimental rat glaucoma. Our reproducible data supported the feasibility of cell-specific
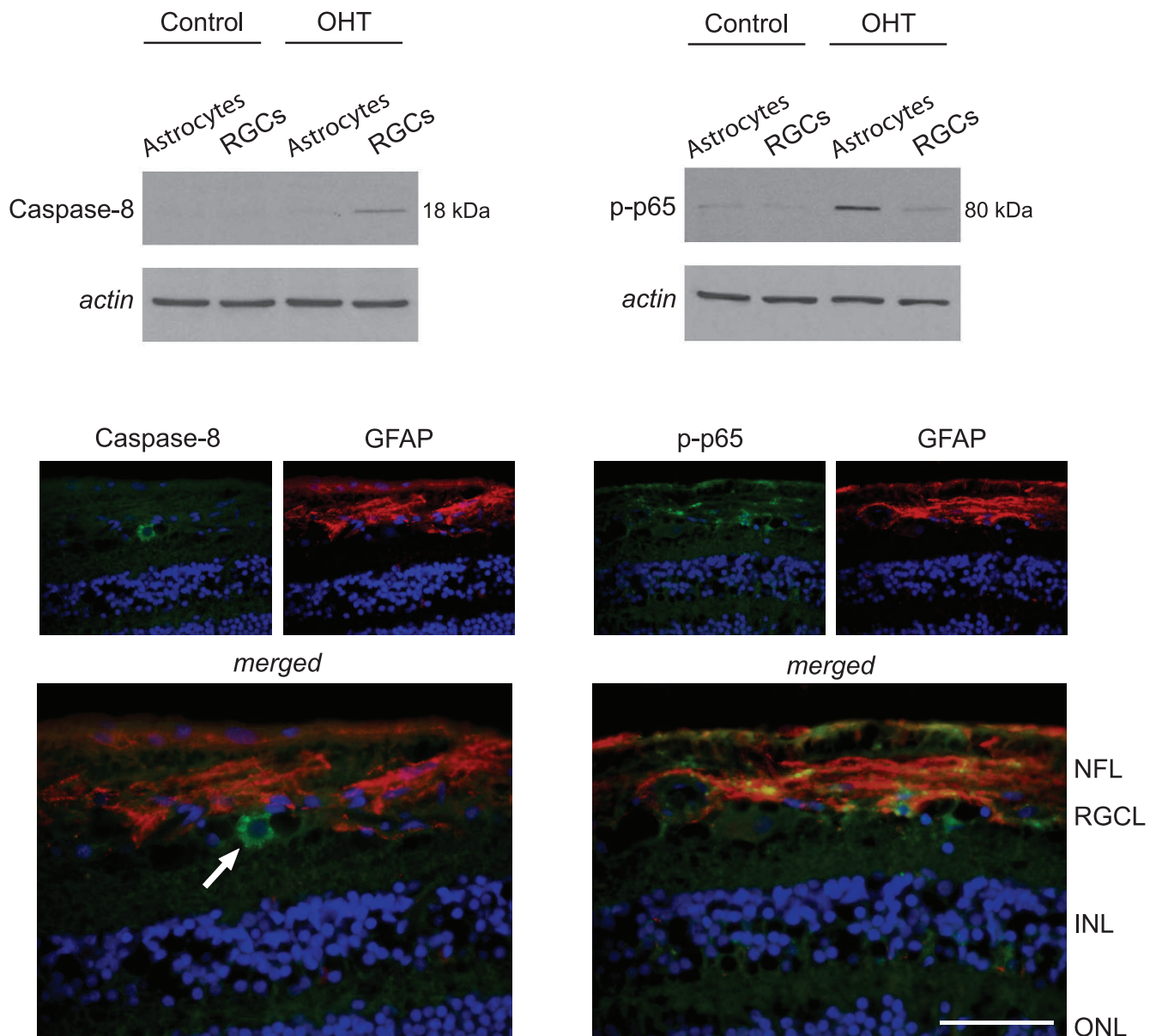
FIGURE 3.   Components of TNF-α/TNFR signaling in OHT astrocytes. Western blot analysis using a cleavage site-specific antibody detected cleaved caspase-8 only in OHT RGCs. However, based on Western blots with a phosphorylation site-specific antibody, p65 (a subunit of NF-κB) activation was detectable most prominently in OHT astrocytes. Immunohistochemistry images show the OHT retina. No immunofluorescence labeling for cleaved caspase-8 (*green*) was detectable in GFAP-positive astroglia, while phospho-p65 (*green*) was localized predominantly to GFAP-positive (*red*) astrocytes. *Arrow* shows a GFAP-negative cell in the RGC layer, exhibiting prominent immunolabeling for cleaved caspase-8. Based on its larger, lighter, and circular nucleus, this GFAP-negative cell in the RGC layer possibly is a RGC. *Blue* corresponds to nuclear DAPI labeling. Data represent three independent sets of analyses with different samples. Scale bar 100 μm.

sampling, and validated the sensitivity of cell-specific analysis to characterize distinct responses of astrocytes during the experimental paradigm. Similar to our previous studies,[15,16,18–20] we used a technique through which target cells are coupled immunologically to magnetic beads and then separated out from the mixed cell population using a magnetic field. This cell isolation process, which can be completed within approximately one hour, presents advantages over alternative techniques. A similar immunomagnetic technique has been used previously for rapid isolation of brain astrocytes with high purity and viability in culture.[28] Cell-specific sampling allows direct analysis of primary astrocytes by avoiding phenotypic changes in tissue cultures. Due to this unique advantage, we had no intention to culture isolated astrocytes in this in vivo study, although we successfully used similarly isolated RGCs in previous in vitro studies.[15,16] Despite appropriate sensitivity to determine differences in protein expression within distinct cell types, it remains unclear whether even a minimum contamination with a few other cell types may be problematic for the analysis of gene expression using these samples. In addition, sufficient sampling of astrocytes for protein analysis (and data validation by immunoblotting) may require large numbers of animals; however, rapidly emerging experimental techniques, ultrasensitive instruments, and new antibodies are expected to improve cell-specific sampling and analysis even further. There is no doubt that assessing cell-specific responses is an ideal way
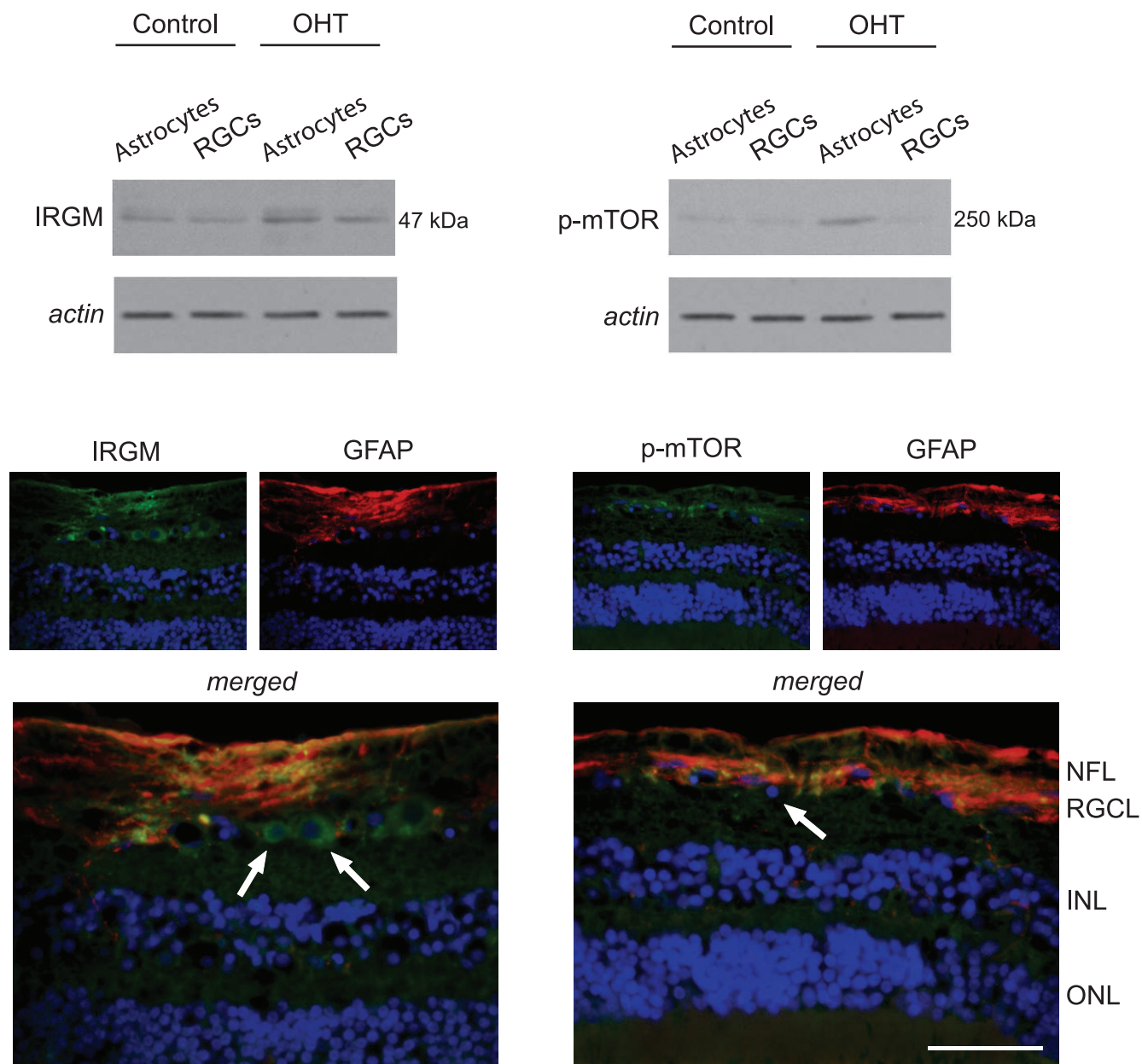
FIGURE 4. Regulation of autophagy signaling in ocular hypertensive astrocytes. Western blot analysis detected prominent up-regulation of IRGM in OHT astrocytes. In addition, probing with a phosphorylation site-specific antibody to mTOR indicated increased immunoreactivity in these samples of OHT astrocytes. Based on immunofluorescence labeling, GFAP-positive (*red*) astrocytes and also some GFAP-negative cells in the RGC layer, likely corresponding to RGCs (*arrows*), exhibited IRGM immunolabeling (*green*) in the OHT rat retina. However, phospho-mTOR (*green*) immunolabeling was detectable predominantly in GFAP-positive astrocytes. *Blue* corresponds to nuclear DAPI labeling. Data represent three independent sets of analyses with different samples. Scale bar 100 μm.

to determine molecular pathways involved in disease pathogenesis, since analysis of whole retina or optic nerve may reflect only the sum of opposing responses from many different cell types. By dissecting the complexity of prior data obtained from whole tissue, the cell-specific analysis enables specific cellular responses to be defined and cell-specific treatment strategies to be developed. Characterization of astrocyte responses can help design treatments to similarly enhance RGC survival in glaucoma and also enable to manipulate astrocyte responses for the gain of RGCs so that neurodestructive consequences may be interfered selectively without compromising glial neurosupportive and homeostatic functions.

Astroglial Components of TNF-α/TNFR Signaling Linked to Neuroinflammatory Responses

Analysis of enriched astrocytes detected predominantly cellular activation and immune/inflammatory responses in ocular hypertensive samples as opposed to various cell death mediators detected in RGCs isolated from the same experimental eyes. Ocular hypertensive astrocytes exhibited NF-κB activation regulating inflammatory consequences of TNF-α/TNFR signaling as discussed later below, but not caspase activation mediating TNF-α-induced apoptosis. Based on our findings, TNF-α/TNFR signaling during glaucomatous neurodegeneration may induce cell death in RGCs but mediate
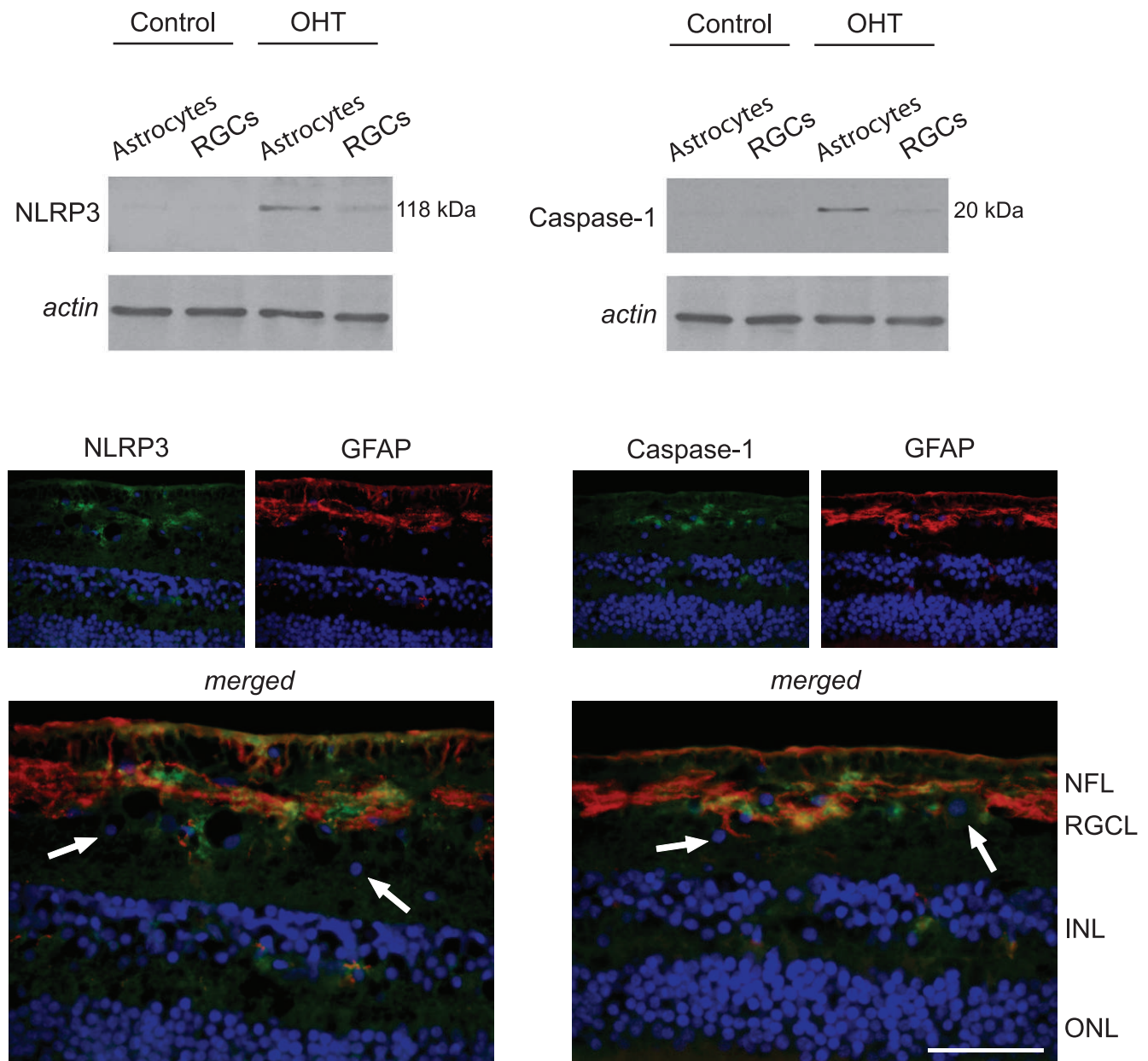
FIGURE 5. Inflammasome components in OHT astrocytes. Western blot analysis using specific antibodies detected a subset of NLRs named NLRP3 and cleaved caspase-1 predominantly in OHT astrocytes. Immunofluorescence labeling of the OHT rat retina indicated prominent localization of NLRP3 and caspase-1 (*green*) in GFAP-positive (*red*) astrocytes. *Arrows* show GFAP-negative cells in the RGC layer exhibiting no detectable immunolabeling for NLRP3 or caspase-1. *Blue* corresponds to nuclear DAPI labeling. Data represent three independent sets of analyses with different samples. Scale bar 100 μm.

immune/inflammatory responses in astrocytes. This is consistent with previous in vitro findings that support a relative resistance of astrocytes, including retinal astrocytes,[15] to death receptor-mediated apoptosis.[29] Our present data suggested that various molecules may regulate cell-specific outcomes of TNF-α/TNFR signaling in glaucoma.

First, ocular hypertensive astrocytes exhibited up-regulated expression of a signal transducer protein involved in the multiprotein signaling complex formed after TNFR binding, namely TNFR-associated death domain protein (TRADD). This multifunctional protein not only is crucial for diverse consequences of TNFR1 signaling but also for other signaling pathways relevant to inflammatory responses.[30] Second, up-regulated proteins in ocular hypertensive astrocytes included a dead domain-containing protein particularly abundant in astrocytes, named phosphoprotein enriched in astrocytes 15 (PEA-15).[31] This astrocyte phosphoprotein directs cytokine outcomes toward survival and protects astrocytes from TNF-α-induced apoptosis by binding dead domain-containing proteins[32,33] and controlling the activity of mitogen-activated protein kinases.[34,35] Another up-regulated protein in ocular hypertensive astrocytes was TNF-α-induced protein 2, a primary response molecule involved in inflammatory processes induced by TNF-α.[36,37] More studies are needed to determine the importance of identified molecules as treatment targets to modulate neurodegenerative inflammation and provide neuroprotection in glaucoma.
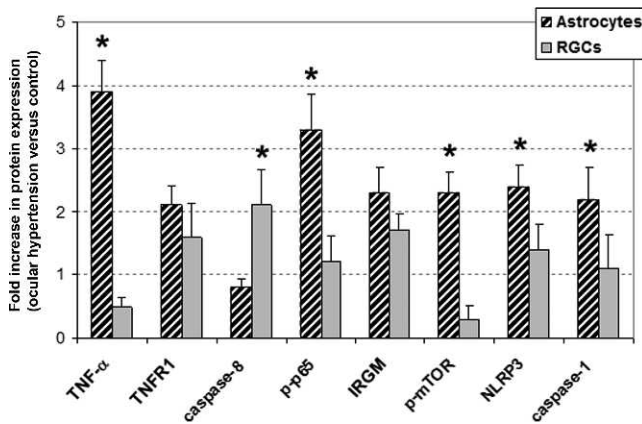
Figure 6. Quantitative Western blot analysis of protein expression in cell-specific samples. Comparison of the beta-actin-normalized intensity values between OHT and control samples supported distinct responses of astrocytes and RGCs. Data represent three independent sets of analyses with different samples. Presented is the mean ± SD fold increase in protein expression, which was determined by comparing the actin-normalized densitometry values of specific immunoreactive bands in OHT samples with those of normotensive controls. *Asterisk* indicates statistically significant difference between astrocyte and RGC responses (Mann-Whitney rank sum test, $P < 0.05$).

NF-κB Activation Regulating Neuroinflammatory Processes in Astrocytes

Our findings support that many of the astrocyte-driven pro-inflammatory processes are mediated by NF-κB. Our proteomic data supporting NF-κB activation in ocular hypertensive astrocytes included NF-κB subunits, p65 (RelA) and p100 (NF-κB2). In addition, immunolabeling with a phosphorylation site-specific antibody detected active p65. In contrast to the canonical pathway through RelA/p50 signaling, the non-canonical pathway of NF-κB signaling upon binding of a small subset of TNFR family members targets predominantly the activation of RelB/p52 complex.[38,39] However, the classical pathway also feeds into the alternative pathway through up-regulation of NF-κB2 expression,[40] and the alternative pathway regulates nuclear localization of RelA besides RelB.[41]

Phosphorylation triggers ubiquitination of inhibitor IκB molecules for proteasomal degradation. The ubiquitination machinery involved in NF-κB activation is regulated by a highly conserved protein complex designated as COP9 signalosome (CSN).[42] The CSN complex interferes with ubiquitination and proteasomal degradation of the inhibitory molecule, IκB, and allows rapid activation of NF-κB.[43] Our data included increased expression of different CSN subunits in ocular hypertensive astrocytes. In a fashion similar to this cytoplasmic control mechanism, NF-κB activation may also be terminated through intranuclear sequestration and degradation.[44] Members of the PDZ and LIM domain proteins, also detected in astrocyte samples, have been shown to be expressed in brain astrocytes[45] and implicated in nuclear regulation of NF-κB activation.[46] Another protein up-regulated in ocular hypertensive astrocytes was a subunit of activating signal cointegrator 1 complex (ASCC). This protein, originally isolated as a transcriptional co-activator of nuclear receptors, is also known to stimulate the activation of NF-κB.[47]

Thus, various molecules appear to be involved in NF-κB activation in ocular hypertensive astrocytes. This redox-sensitive transcription factor is a master regulator of inflammatory responses and secondary injury processes in the brain,[48,49] and inactivation of astroglial NF-κB reduces inflam-

matory environment after ischemic injury and promotes RGC survival.[50] Findings of our recent study also have supported NF-κB activation in the glaucomatous human retina.[5]

In addition to NF-κB pathway controlling the transcription of immune mediators, the c-Jun N-terminal protein kinase/activator protein 1 (AP-1) signaling can stimulate inflammation through the activation of gene transcription.[51] The AP-1, also up-regulated in ocular hypertensive astrocytes, is among the best characterized inducible DNA-binding proteins involved in a number of cellular processes linked to inflammatory responses of brain astrocytes.[52]

We also detected increased expression of various signal transducers and activators of transcription (STAT) in ocular hypertensive astrocytes. The janus kinase (JAK)/STAT signaling, along with the NF-κB signaling, has received a growing attention as key regulators of cytokine-mediated inflammation[53,54] and promising targets for immunomodulation.[55] Various components of the JAK/STAT signaling pathway recently have been indicated in the glaucomatous human retina,[5] ocular hypertensive rat retina,[56] and optic nerve.[57]

Our MS/MS data also included glia maturation factors that mediate inflammation in the central nervous system and exhibit up-regulation in neurodegenerative diseases,[58] and the macrophage migration inhibitory factor (MIF), which is expressed by retinal glia[59] and has an important amplifying role in cytokine-mediated inflammatory diseases.[60]

Cell-Specific Regulation of Autophagy

Molecular responses of ocular hypertensive astrocytes included up-regulation of IRGs that are key mediators of the host resistance to intracellular pathogens with important links to autophagy.[61,62] We also detected activation of a serine/threonine protein kinase, mTOR, which is an upstream negative regulator of autophagy signaling,[63,64] and ATGs, which are involved in the execution stages of autophagy.[65]

Autophagy is a physiological cell-autonomous defense mechanism enabling cells to digest their own cytosol, remove toxic products, and eliminate defective or surplus organelles. This cytoplasmic homeostasis pathway allows cells to survive nutrient depletion or the absence of growth factors; however, under specific conditions, it also may determine cell death.[66,67] Although the autophagic pathway is activated in RGCs following optic nerve transaction[68] or retinal ischemia,[69] the role of autophagy in glaucomatous neurodegeneration is unknown. Many questions raised from our findings motivate further research to clarify the importance and regulation of autophagy and its crosstalk with RGC apoptosis in glaucoma. Autophagy can be induced by cytokines, including TNF-α,[70,71] which also up-regulates IRGs in astrocytes,[72] while NF-κB activation represses TNF-α-induced autophagy through the activation of autophagy inhibitor, mTOR.[73] Since NF-κB was activated predominantly in glaucomatous astrocytes, it would be interesting to determine whether NF-κB-dependent activation of mTOR controls the balance between activator and inhibitor pathways of autophagy in astrocytes and other retinal cell types.

It also is interesting that autophagy has recently been recognized as a biological pathway broadly associated with innate and adaptive immunity.[74–76] By eliminating intracellular microbial pathogens, and regulating T and B cell homeostasis, a properly functioning autophagy helps prevent inflammation and autoimmunity. However, when functioning aberrantly, it may contribute to immunogenic neurodegenerative human diseases. Not only does it act as an innate effector downstream of toll-like receptors (TLRs),[77] but autophagy can also deliver danger-associated molecular patterns to endosomal TLRs[78] and process cytosolic autoantigens for presentation on MHC class II molecules.[79–81] In conjunction with earlier studies of increased
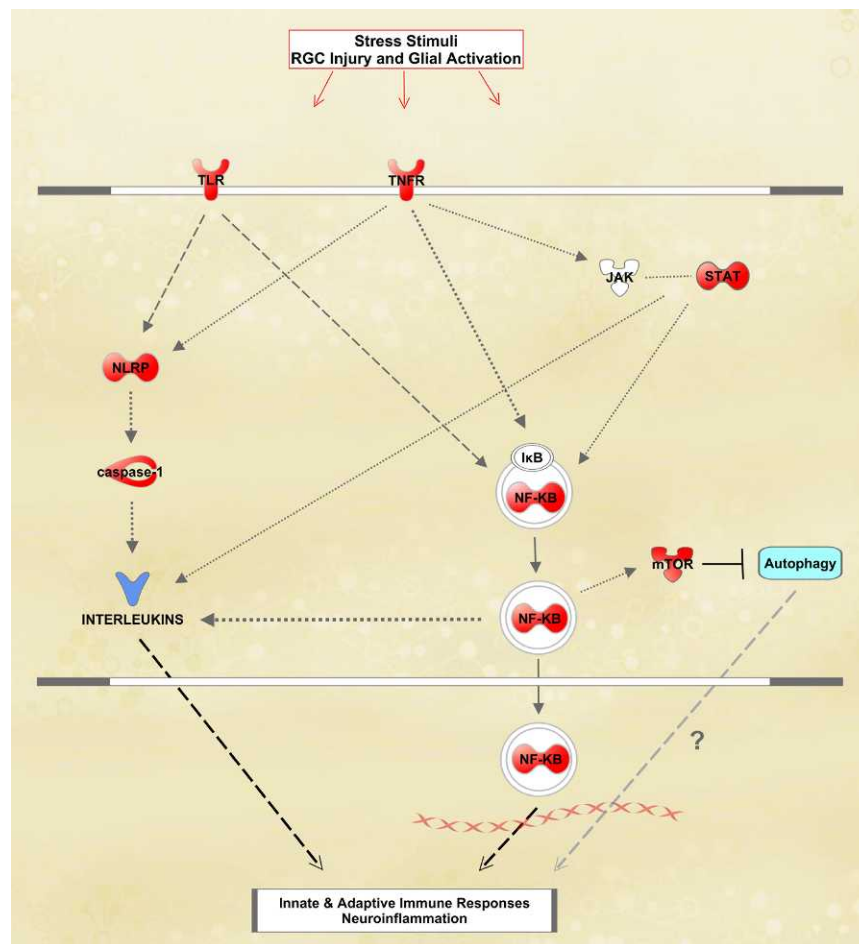
Figure 7. Astrocyte proteins linked to inflammatory responses in OHT samples. In this simplified network generated by The Ingenuity Pathways Analysis, the proteins shown in *red* exhibited significantly increased expression in OHT samples relative to normotensive controls.

serum autoantibodies in glaucoma, our findings bring about additional questions stimulating further research. For example, it is tempting to determine whether the autophagy-mediated route for efficient presentation of autoantigens may have a role in autoantibody generation in glaucoma. Retinal antigens identified so far, which are not limited to RGC proteins, possibly may reflect enhanced presentation of autoantigens on various cell types undergoing autophagy. With respect to chaperone-mediated autophagy,[82] heat shock protein antibodies commonly present in the glaucomatous sera may particularly be relevant to autophagy-mediated immunity.

Neuroinflammatory Responses of Astrocytes Linked to Inflammasome

Our data also supported inflammasome activation in ocular hypertensive astrocytes. Upon sensing intrinsic danger signals as well as microbial components, this cytosolic multiprotein complex promotes proteolytic activation of pro-interleukins and secretion of mature cytokines.[83,84] This innate mechanism mediates neuroinflammation in the brain, and its therapeutic neutralization reduces the damaging effects of post-traumatic brain inflammation.[85,86] Similar to our recent study of human glaucoma,[5] the present study detected various inflammasome components in experimental rat glaucoma. Up-regulated astrocyte proteins in ocular hypertensive samples included a specific NLRP adaptor for inflammasome assembly, apoptosis-associated speck-like protein containing a caspase recruitment

domain (ASC).[83,84] Also detected in ocular hypertensive astrocytes was up-regulation and proteolytic activation of the inflammatory caspase, caspase-1. In addition to potassium efflux, amyloid-β, and pannexins,[87] oxidative stress, evident in human glaucoma,[3,88] has been implicated in inflammasome formation.[89-91] Despite some controversies,[92] caspase-1 activation is sensitive to alterations in the cellular redox balance.[93] Emerging evidence also provides links between inflammasome and autophagic pathways.[94]

In summary, our findings introduced a cell-specific proteomic approach and validated its sensitivity to identify astrocyte responses in experimental rat glaucoma. Various pathways, including pro-inflammatory TNF-α/TNFR signaling, NF-κB activation, and inflammasome (along with the TLR signaling[4] and complement activation[3]), appear to be co-players of inflammatory responses mediated by ocular hypertensive astrocytes and may represent key treatment targets for immunomodulation. Further research using focus-in strategies and functional testing are expected to generate a greater understanding of target molecules for cell-specific treatments in glaucoma.

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