Glaucomatous Tissue Stress and the Regulation of Immune Response through Glial Toll-like Receptor Signaling

Cheng Luo,[1] *Xiangjun Yang,*[1] *Angela D. Kain,*[2] *David W. Powell,*[2] *Markus H. Kuehn,*[3] *and Gülgün Tezel*[1,4]

Purpose. To determine the regulation of immune system activity associated with Toll-like receptor (TLR) signaling in glaucoma.

Methods. Retinal protein samples obtained from human donor eyes with (n = 10) or without (n = 10) glaucoma were analyzed by a quantitative proteomic approach involving mass spectrometry. Cellular localization of TLR2, -3, and -4 was also determined by immunohistochemical analysis of an additional group of human donor eyes with glaucoma (n = 34) and control eyes (n = 20). In addition, in vitro experiments were performed in rat retinal microglia and astrocytes to determine glial TLR expression and immunoregulatory function after exposure to exogenous heat shock proteins (HSPs) and H_2O_2-induced oxidative stress.

Results. Proteomic analyses of the human retina detected expression and differential regulation of different TLRs in glaucomatous samples. Parallel to the upregulation of TLR signaling, proteomic findings were also consistent with a prominent increase in the expression of HSPs in glaucoma. Immunohistochemical analysis supported upregulated expression of TLRs on both microglia and astrocytes in the glaucomatous retina. In vitro experiments provided additional evidence that HSPs and oxidative stress upregulate glial TLR and MHC class II expression and cytokine production through TLR signaling and stimulate proliferation and cytokine secretion of co-cultured T cells during antigen presentation.

Conclusions. The findings of this study support the upregulation of TLR signaling in human glaucoma, which may be associated with innate and adaptive immune responses. In vitro findings showed that components of glaucomatous tissue stress, including upregulated HSPs and oxidative stress, may initiate the immunostimulatory signaling through glial TLRs. (*Invest Ophthalmol Vis Sci.* 2010;51:5697–5707) DOI:10.1167/iovs.10-5407

From the Departments of [1]Ophthalmology and Visual Sciences, [2]Medicine-Clinical Proteomics Center, and [4]Anatomical Sciences and Neurobiology, University of Louisville School of Medicine, Louisville, Kentucky; and the [3]Department of Ophthalmology and Visual Sciences, the 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.

Increasing evidence obtained from clinical and experimental studies supports a prominent immune response during glaucomatous neurodegeneration.[1–3] Experimental findings of in vivo and in vitro studies also collectively suggest that, if there is a failure in the regulation of immune response, different components of innate and adaptive immunity may create a stimulus that facilitates the progression of neurodegenerative injury at different compartments of retinal ganglion cells (RGCs). For example, increased glial production of neurotoxic cytokines,[4,5] T cell-mediated[6] and autoantibody-mediated[7] cytotoxicity, and uncontrolled complement attack[8,9] may all lead to neurodegenerative consequences.

Stress response of the glaucomatous retina and optic nerve head during glaucomatous neurodegeneration includes an upregulated expression of heat shock proteins (HSPs). The upregulation of HSPs is thought to be an intrinsic adaptive mechanism to protect stressed cells from further damage and to facilitate tissue repair.[10] As suggested by the increased titers of HSP antibodies detected in the glaucomatous patient serum,[11–13] besides their functions in intrinsic protective mechanisms, HSPs may also serve as an immunostimulatory signal to the immune system to detect and eliminate stressed cells. Similar to observations in experimental animal models of glaucoma, glial cells in the glaucomatous human retina and optic nerve head, including both microglia and macroglia, rapidly respond to any disturbance of tissue homeostasis by exhibiting an activated phenotype.[14,15] The glial activation response in human glaucoma includes the activation of glial immunoregulatory functions, such as the activation of cytokine production[5] and antigen-presenting abilities.[14,15] As supported by in vitro observations, these resident immunoregulatory cells can detect the danger signals arising from stressed cells and initiate an immune response.[16] However, the molecular mechanisms underlying the glia-mediated regulation of immune response are unclear.

Mammalian Toll-like receptors (TLRs) are cellular pattern-recognizing receptors that recognize the molecular patterns of pathogens, such as bacterial lipopolysaccharide (LPS) and viral double-stranded RNA. Although they are firmly established as pathogen receptors, growing evidence supports the notion that TLRs can also sense nonpathogen stimuli, thereby mediating the immune response. Over the past decade, it has become clear that one group of nonpathogenic intrinsic ligands of TLRs is the HSPs,[17,18] with important links to the mediation of neuroinflammation and neurodegeneration in the central nervous system.[19,20] In this study, we sought to determine the expression of TLRs in human glaucoma and their roles in glia-mediated immune activity in vitro. Proteomic and immunohistochemical findings revealed an upregulation of some TLRs in glaucomatous human donor eyes, and in vitro findings in cell cultures of rat retinal microglia and astrocytes supported the idea that HSPs and oxidative stress can stimulate immune

activity through glial TLR signaling. These in vitro findings have motivate further studies to determine whether components of glaucomatous tissue stress, including upregulated HSPs[10] and oxidative stress,[21] may similarly activate glial TLRs and initiate immunostimulatory signaling during glaucomatous neurodegeneration.

Materials and Methods

Experimental Design

For proteomic analysis, retinal protein samples were obtained from human donor eyes, with or without glaucoma. Cellular localization of TLRs, including TLR2, -3, and -4, was also determined in histologic sections of the retina obtained from an additional group of glaucomatous and nonglaucomatous human donor eyes. All human donor eyes were handled according to the tenets of the Declaration of Helsinki. In addition, we performed in vitro experiments to better understand glial TLR signaling. TLR expression and immunoregulatory function were studied in primary cultures of microglia and astrocytes derived from rat retinas. To simulate potential intrinsic ligands of TLRs in glaucoma, we used exogenous HSPs and H_2O_2-induced oxidative stress in the presence and absence of specific treatments. All animals used in the in vitro experiments 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.

Human Donor Eyes

Retinal protein samples obtained from 10 human donor eyes with glaucoma (age, 84.7 ± 8 years) and 10 eyes without glaucoma (age, 83.7 ± 7 years) were individually analyzed. Retinal tissue punches were collected as previously described[22] within less than 6 hours after death (average postmortem time, 4.33 hours for the glaucomatous eyes; 4.53 hours for the control eyes). All the glaucomatous donor eyes had primary open-angle glaucoma with high intraocular pressure, which was well documented by intraocular pressure readings, optic disc assessments, and visual field tests. Protein samples were 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, as previously described.[22–24]

Additional human donor eyes, including 38 with a diagnosis of glaucoma (age, 76.8 ± 11 years) and 30 eyes of donors without glaucoma (age, 71.0 ± 15 years), were used for immunohistochemical analysis. All the eyes were fixed within 12 hours after death and processed for 5-μm paraffin-embedded sagittal tissue sections. Detailed information on donor demographics and clinical data on glaucomatous donor eyes have recently been published.[21]

Proteomic Analysis

Trypsin-digested protein samples were analyzed through a gel-free approach involving two-dimensional capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS), as previously described.[22,25] The acquired MS/MS spectra were analyzed with a program (Sequest Sorcerer; Sage-N Research, Inc., San Jose, CA) that was set up to search a FASTA-formatted human protein database (Human RefSeq; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, at www.ncbi.nlm.nih.gov/locuslink/refseq/) with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.2 Da. A specific software (Scaffold, ver. 3.0; Proteome Software, Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at a greater than 95% probability, as specified by the Peptide Prophet algorithm.[26] Protein probabilities were assigned by the Protein Prophet algorithm,[27] and protein identifications were accepted if they could be established at a greater than 99% probability and contained at least two identified peptides. The abundance of each identified protein was determined by normalizing the number of unique spectral counts matching the protein by its predicted molecular mass. This value has been termed a protein abundance factor.[25,28] The Mann-Whitney rank sum test was used to determine the significant difference in protein expression between glaucomatous and control samples.

A pathway analysis system (Ingenuity Pathways Analysis; Ingenuity Systems, Mountain View, CA) was used for the bioinformatics analysis of the proteomic data to define functional patterns within the human retinal proteome composition, as previously described.[25,29] Canonical pathway analyses identified the pathways from the Ingenuity Systems library of canonical pathways that were most significant in the data set. To determine the likelihood that a given canonical pathway is associated with the proteins in the data set by random chance, *P* values were calculated by using the right-tailed Fisher's exact test.

Morphologic Analysis

We determined the cellular localization of TLRs by performing immunohistochemical analysis of histologic sections of the retina. All procedures were similar to those published.[21,22,24,30] Monoclonal antibodies to TLR2, -3, and -4 (2 μg/mL; Assay Designs, Ann Arbor, MI) served as the primary antibodies. In addition, antibodies against CD11b (1:100; Abcam, Cambridge, MA) or glial fibrillary acidic protein (GFAP; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) were used to identify microglia and astrocytes during double immunofluorescence labeling. 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. For each procedure, at least four histologic sections were used from each eye, including those obtained from the superior and inferior half of the retina. All slides were masked as to donor identity. Negative controls were performed by replacing the primary antibody with serum or by incubation with each primary antibody followed by the inappropriate secondary antibody, to determine that each secondary antibody was specific to the species that it was raised against.

Cell Culture Experiments

Rat (Brown Norway; Harlan, Indianapolis, IN) retinal microglia cultures were prepared as previously described.[24] Briefly, mixed retinal cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) supplemented with 10% serum, 1 ng/mL granulocyte/macrophage colony-stimulating factor (Sigma-Aldrich, St. Louis, MO), and antibiotics. Microglia were detached by vigorous pipetting after incubation with Ca^{2+}-Mg^{2+}-free Hanks' balanced salt solution (Sigma-Aldrich) containing 0.2% EDTA and 5% serum at 4°C for 1 hour.

Rat retinal astrocyte cultures were prepared by using the retinal cells depleted for microglia and ganglion cells according to the two-step immunomagnetic selection process previously described.[4,24] This unselected fraction of retinal cells containing macroglial cells was cultured in a medium that does not allow residual neurons to survive (Dulbecco's minimum essential medium, 10% fetal bovine serum, 2 mM glutamine, 1 mM Na-pyruvate, and antibiotics).[4] During the experimental period, glial cell cultures were incubated in a serum-free medium containing DMEM; 1.3% bovine albumin fraction V; 1 μL/mL of an insulin, human transferrin, and selenic acid–containing culture supplement (Premix; BD Biosciences, San Diego, CA); and antibiotics.

Syngeneic T-cell cultures derived from Brown Norway rats were also prepared as described in previous studies.[24,31] Briefly, T cells isolated from the spleen and lymph nodes by passage through a nylon wool column were stimulated with 10 μg/mL antigen (myelin basic protein) in the presence of irradiated spleen cells to become antigen-presenting cells. After 2 days, activated lymphoblasts were isolated by gradient centrifugation in a separation medium (Lymphoprep; Robbins Scientific, Mountain View, CA) and cultured in RPMI-1640 medium (Mediatech, Herndon, VA) supplemented with 20 U/mL interleukin-2 (R&D Systems, Minneapolis, MN).

During the experimental period, the glial cells were incubated in the absence and presence of recombinant HSPs, including recombinant HSP60 or -70 containing low endotoxin (10 μg/mL; Assay Designs), or H_2O_2 (50 μM; Sigma-Aldrich) for 48 hours. Any effect from possible endotoxin contamination was excluded in the control experiments by using the HSPs pretreated with proteinase K or boiled for 20 minutes before incubation. Positive controls included interferon-γ (IFN-γ; 20 ng/mL; R&D Systems), an inducer of glial antigen presentation,[24] and purified *Escherichia coli* LPS (100 ng/mL; Sigma-Aldrich), a TLR4 ligand.[17,18] In the in vitro experiments, we also used specific treatments to inhibit MyD88 or TIRAP (50 μM; Imgenex, San Diego, CA), both of which are adaptor proteins involved in TLR signaling. The MyD88 inhibitor was a cell-permeable peptide containing a sequence from the MyD88 TIR homodimerization domain that blocks MyD88 homodimerization, thereby inhibiting MyD88-dependent TLR signaling.[32,33] The second inhibitor was a cell-permeable peptide that contains a TIRAP sequence and functions as a TIRAP decoy by binding to TIR-interacting domains on specific TLRs, thereby blocking the TIR–TIR domain interaction between TIRAP and the receptor.[34,35] These inhibitor treatments were applied 24 hours before the incubation with HSPs or H_2O_2. Control cultures, prepared from cells of an identical passage, were simultaneously incubated in the absence of these treatments. At the end of the experimental period, glial expression of TLRs and MHC class II was determined by Western blot analysis, and TNF-α levels were measured in the glial conditioned medium by enzyme-linked immunosorbent assay (ELISA). In addition, the antigen-presenting function was determined by coculturing glial cells with T cells, as previously described.[24] At the end of the 48-hour co-incubation period, T-cell proliferation and cytokine secretion were determined. Glial cells were treated with mitomycin C (100 μg/mL; Sigma-Aldrich) for 1 hour, before they were mixed with the T cells, to exclude the possibility that the cytokine production in co-cultures would be from glial cells.[36] Wells without antigen were simultaneously processed as the control. In addition, untreated co-culture wells and control wells containing T cells cultured alone were included in each plate. Cell viability was determined with a live/dead kit containing calcein AM (Molecular Probes) as previously described.[4,16,22,37] All in vitro experiments were performed in triplicate wells and independently repeated three times in separate cell cultures. An integrated value was obtained for treated and untreated cultures after background subtraction using negative controls. Statistical differences between treated and untreated cultures were analyzed by Mann-Whitney rank sum test, using these normalized values.

Western Blot Analysis

Immunoblot analysis was performed as previously described,[22–24] with the specific antibodies to TLRs described earlier and a monoclonal antibody to MHC class II (1:100; Novus Biologicals, Littleton, CO). In addition, a β-actin antibody (Sigma-Aldrich) was used to reprobe the stripped immunoblots for loading and transfer control. The secondary antibody incubation was performed with a specific IgG conjugated with horseradish peroxidase (1:2000; Sigma-Aldrich). In negative control experiments, the primary antibody was omitted. After normalization to β-actin, the average band intensity obtained from untreated samples was used to calculate the ratio of change in protein expression in treated samples.

Enzyme-Linked Immunosorbent Assay

A commercial kit (R&D Systems) was used to measure TNF-α levels in the conditioned culture medium by the quantitative sandwich ELISA technique.[16] The data are presented as the mean change ratio ± SD, which was calculated by comparing the normalized values in treated cultures with those in the untreated control cultures.

Lymphocyte Proliferation Assay

The proliferation of T cells was measured as described in previous studies.[24,38] Briefly, at the end of the incubation period, 0.5 μCi [^{3}H]-thymidine/well was added to culture plates for 6 hours. In addition to the control cultures described earlier, wells that were not incubated with [^{3}H]-thymidine served as a negative control. The cells were harvested and assessed by microplate scintillation counter (Packard Instrument/Perkin Elmer, Boston, MA) for isotope incorporation. The mean (±SD) change in proliferative response was calculated by comparing the treated versus untreated cultures after background subtraction, using the negative controls.

Results

Differential Expression of TLRs in the Glaucomatous Human Retina

Proteomic analysis of human retinal protein samples obtained from donor eyes with or without glaucoma detected the expression of different TLRs. Table 1 shows a list of the identified retinal proteins and their differential expression in human glaucoma. Our studies using proteomic analysis techniques similarly detected differential expression of TLRs in retinal protein samples obtained from rat eyes with experimental glaucoma; however, only the human data of particular relevance are presented herein. As shown in Table 1, based on the quantitative LC-MS/MS analysis, differentially expressed proteins in human glaucoma also included an important adaptor protein of the TLR signaling, MyD88.

Pathways analysis (Ingenuity Systems) defined the TLR signaling pathway among the canonical pathways significantly associated with our human retinal proteomic data (right-tailed Fisher's exact test; $P < 0.05$). Figure 1 shows the integration of the identified proteins into the canonical pathway of TLR signaling in Ingenuity's Knowledge Base. As shown in Figure 1, bioinformatics analysis of our high-throughput proteomic data linked many other proteins identified in the human retinal proteome to the TLR signaling leading to cytokine production, cell death, and immune response. Note that many signaling molecules involved in this pathway (but not shown in Table 1) are not specific to TLR signaling.

Immunohistochemical analysis was also performed to determine the cellular localization of TLRs in the human retina. Specific antibodies to TLRs (TLR2, -3, and -4) and cell markers

Table 1. Proteomic Analysis of the Retinal Proteins in Human Glaucoma

RefSeq Accession	Protein Acronym	Change Ratio (Glaucoma/Control)
NP_003255	Toll-like receptor 2	2.9*
NP_612564	Toll-like receptor 4 isoform A	3.6*
NP_057646	Toll-like receptor 7	1.6*
NP_057694	Toll-like receptor 8 isoform 1	1.4
NP_112218	Toll-like receptor 10 precursor	0.9
NP_002459	MyD88	Detected only in glaucoma

Individual retinal protein samples obtained from human donor eyes with ($n = 10$) or without ($n = 10$) glaucoma were used in quantitative LC-MS/MS analysis. Identified proteins included different TLRs and an adaptor protein of the TLR signaling, MyD88. Note that this adaptor protein was detectable only in glaucomatous samples. 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. RefSeq is provided in the public domain by the National Center for Biotechnology Information. Available at www.ncbi.nlm.nih.gov/locuslink/refseq/.

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

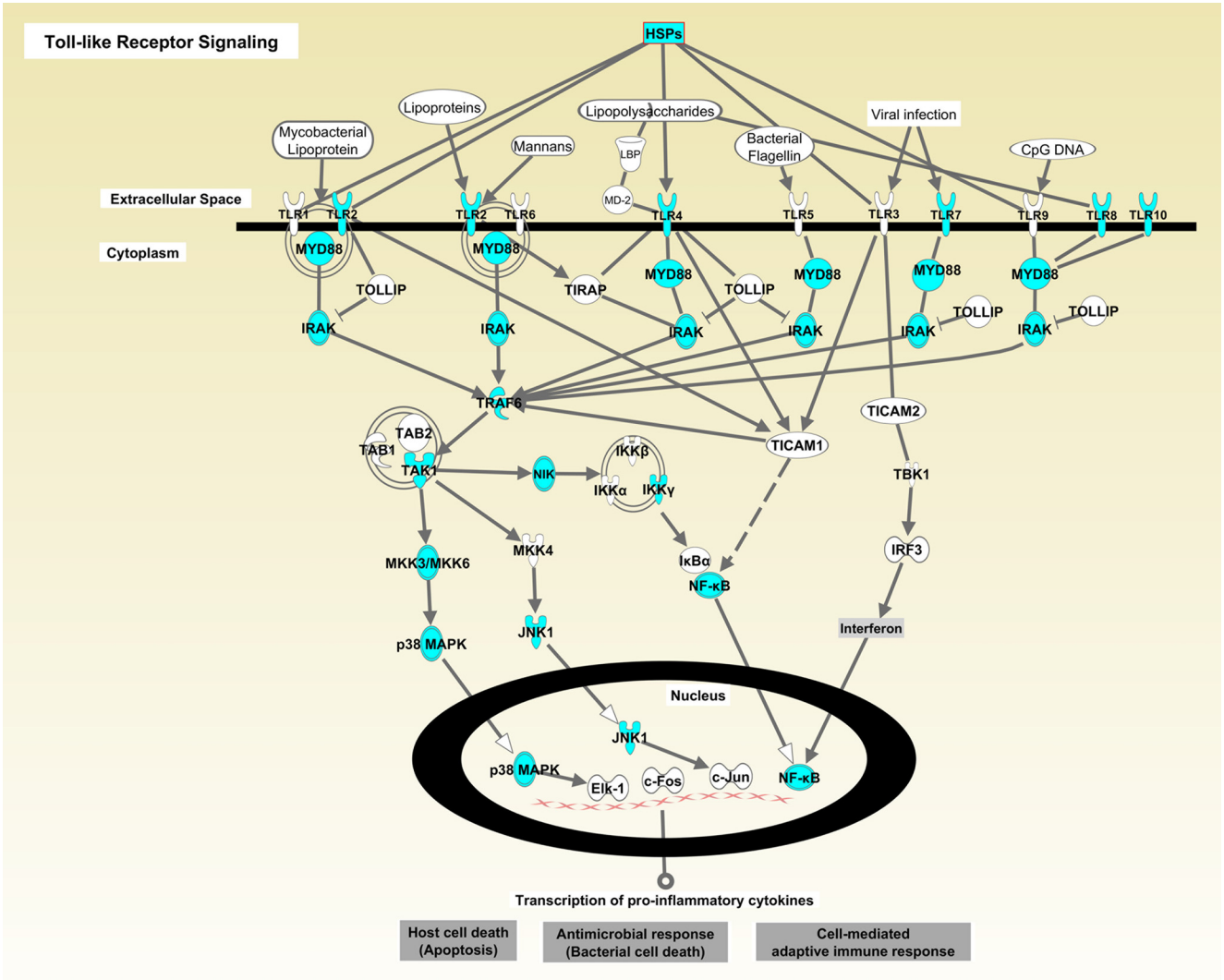

FIGURE 1. Integration of the identified proteins into the canonical pathway of TLR signaling. Using the bioinformatics analysis tools (Ingenuity Pathways Knowledge Base), the TLR signaling pathway was defined among the canonical pathways significantly associated with our high-throughput human proteomic data (right-tailed Fisher's exact test; $P < 0.05$). Proteins shown in *blue* are those identified by the LC-MS/MS analysis of human retinal proteins, which exhibited an upregulation or were detectable only in glaucomatous samples (detailed information for abbreviated proteins is available at http://www.ncbi.nlm.nih.gov/protein).

for glial cell types were used for double immunofluorescence labeling of the retina sections obtained from glaucomatous and nonglaucomatous donor eyes. Consistent with proteomic findings, double immunofluorescence labeling detected TLRs on both retinal microglia (Fig. 2) and astrocytes (Fig. 3). Although retinal microglia exhibited prominent immunolabeling for all three TLRs studied, TLR3 immunolabeling was predominant on astrocytes compared with the immunolabeling of these cells for TLR2 or -4.

Since HSPs are now considered to be intrinsic ligands of TLRs,[17,18] we also sought to document the retinal HSP response in the glaucomatous human retina. Our proteomic data, obtained from glaucomatous and nonglaucomatous samples, were consistent with a prominent upregulation of HSPs, parallel to the upregulation of TLR signaling. As shown in Table 2, on the basis of the quantitative LC-MS/MS analysis, the expression level of many HSPs exhibited a more than twofold increase in glaucomatous samples relative to that in the control samples, or some of them were detectable only in the glaucomatous retina. Upregulated HSPs in human glaucoma included HSP60 and -70, which were further studied in in vitro experiments.

Glial TLR Signaling and Immunoregulatory Functions In Vitro

In a series of in vitro experiments, we used rat retinal microglia and astrocyte cultures to determine glaucomatous stress-related ligands of the glial TLR signaling. In these studies, HSPs, including HSP60 and -70, and also H_2O_2-induced oxidative stress, stimulated the immunoregulatory functions of glial cells through TLRs (Fig. 4). Western blot analysis of cultured cells detected the expression of TLRs, including TLR2, -3, and -4, in both microglia and astrocyte cultures. The expression of TLR2, -3, and -4 was prominently detectable in microglia; however, the expression of TLR3 was predominant in astrocytes (Fig. 4C). Exposure of glial cells to exogenous HSPs did not cause a significant alteration in cell survival, and the concentration of H_2O_2 that was used resulted in glial cell survival rates of >80% of untreated cultures at 48 hours. However, as shown in Figure 4C, exposure of glial cells to HSP60, -70, and H_2O_2 resulted in a more than twofold increase in TLR expression.

Parallel to the upregulation of TLRs, glial cytokine (TNF-α) secretion was increased, and MHC class II expression was upregulated after exposure of cultured glia to HSPs or H_2O_2.

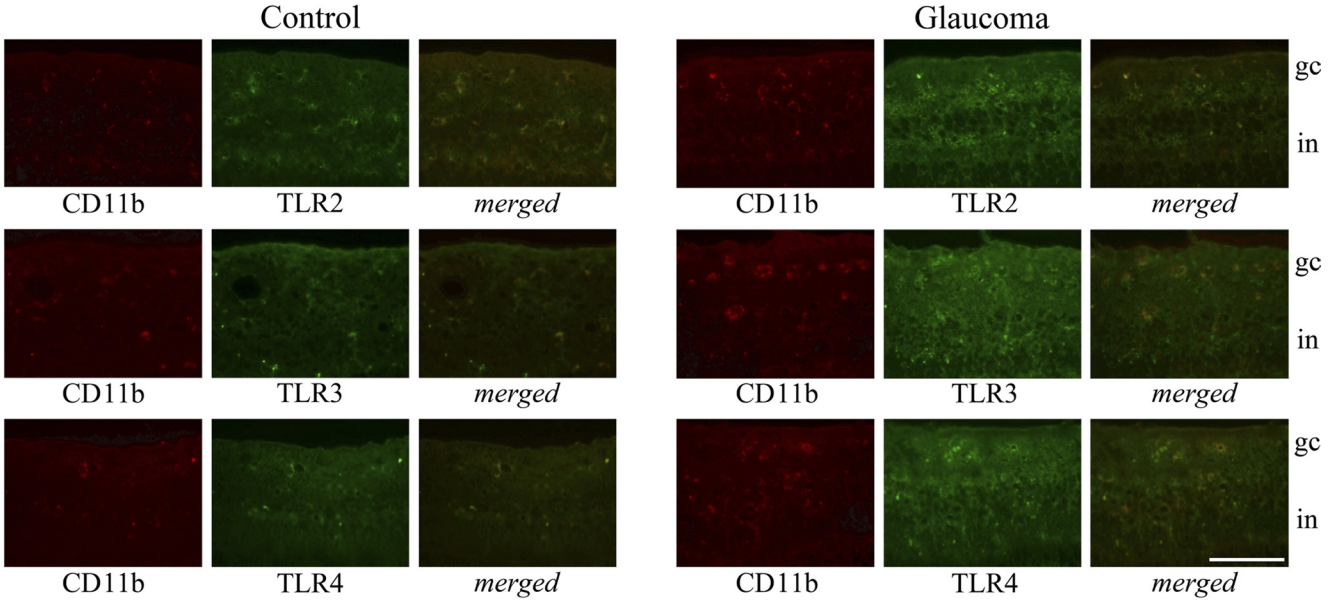

FIGURE 2. Immunohistochemical analysis of the cellular localization of TLRs in the human retina. Images show double immunofluorescence labeling of human retina sections with specific antibodies to TLR2, -3, or -4 (*green*), and CD11b (*red*), a marker for microglial cells. Immunolabeling for all three TLRs was localized to microglia. gc, ganglion cells; in, inner nuclear layers. Scale bar, 100 μm.

Control experiments using IFN-γ treatment resulted in a more than sixfold increase in glial cytokine secretion and MHC class II expression. When microglial cells were exposed to HSPs or H_2O_2, TNF-α secretion (Fig. 4D) and MHC class II expression (Fig. 4F) exhibited a more than fourfold increase. Astrocytes exposed to HSPs or H_2O_2 also exhibited an increase in cytokine secretion (approximately threefold; Fig. 4E) and MHC class II expression (more than fourfold; Fig. 4F). Specific treatments inhibiting adaptor proteins of the TLR signaling, MyD88 and TIRAP, resulted in a more than 60% decrease in HSP- or oxidant-induced stimulation of microglial cytokine secretion and MHC class II expression (Mann-Whitney rank sum test; $P < 0.01$). Although a treatment effect was detectable, TIRAP treatment did not result in a statistically significant change in the stimulated cytokine production or MHC class II expression of astrocytes (Mann-Whitney rank sum test; $P > 0.05$). However, MyD88 treatment resulted in a significant decrease in cytokine production and MHC class II expression of astrocytes exposed to HSP60 or -70 (Mann-Whitney rank sum test, $P < 0.01$). Control experiments with the HSPs pretreated with proteinase K or boiled for 20 minutes before incubation resulted in no prominent stimulation of glial cytokine secretion or MHC class II expression over the untreated control (Mann-Whitney rank sum test, $P > 0.05$). This observation confirmed that the data obtained from HSP-treated cultures were not affected by endotoxin contamination.

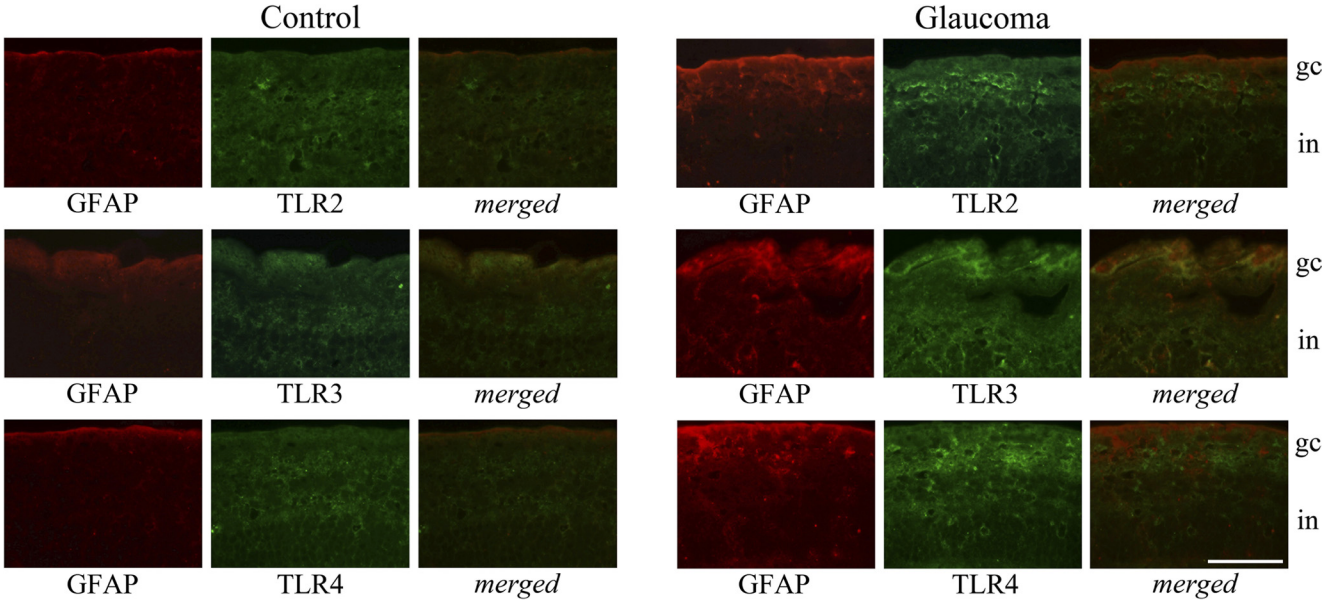

FIGURE 3. Immunohistochemical analysis of the cellular localization of TLRs in the human retina. Images show double immunofluorescence labeling of human retina sections with specific antibodies to TLR2, -3, or -4 (*green*), and GFAP (*red*), a marker for astrocytes. TLR3 immunolabeling exhibited a prominent localization to retinal astrocytes. gc, ganglion cells; in, inner nuclear layers. Scale bar, 100 μm.

TABLE 2. Proteomic Analysis of the Retinal Proteins in Human Glaucoma

RefSeq Accession	Protein Acronym	Change Ratio (Glaucoma/Control)
XP_370704	Similar to 10 kDa heat shock protein	0.6
NP_000385	Crystallin, alpha A	2.1*
NP_001876	Crystallin, alpha B; heat shock 20 kDa like-protein	3.2*
NP_149971	Heat shock protein, alpha-crystallin-related, B9	0.3
NP_001531	Heat shock 27 kDa protein 1	6.4
NP_001530	DnaJ (Hsp40) homolog, subfamily A, member 1; DNAJ-like heat shock protein 2	Detected only in glaucoma
NP_008965	DnaJ (Hsp40) homolog, subfamily B, member 4; DnaJ-like heat shock protein 40	0.5
NP_005485	DnaJ (Hsp40) homolog, subfamily B, member 6 isoform b	Detected only in glaucoma
NP_006251	DnaJ (Hsp40) homolog, subfamily C, member 3	Detected only in glaucoma
NP_005519	DnaJ (Hsp40) homolog, subfamily C, member 4	Detected only in glaucoma
NP_002147	Heat shock 60-kDa protein 1	5.6*
NP_005518	Heat shock 70-kDa protein 1-like	2.9*
NP_005337	Heat shock 70-kDa protein 1B	Detected only in glaucoma
NP_068814	Heat shock 70-kDa protein 2	5.4*
NP_002145	Heat shock 70-kDa protein 4	4.1*
NP_055093	Heat shock 70-kDa protein 4-like	0.2
NP_005338	Heat shock 70-kDa protein 5 (78 kDa glucose-regulated protein)	5.2*
NP_002146	Heat shock 70-kDa protein 6 (HSP70B′)	Detected only in glaucoma
NP_694881	Heat shock 70-kDa protein 8 isoform 2; heat shock cognate protein 54	−1.0
NP_079291	Heat shock 70-kDa protein 12A	1.0
NP_008879	Heat shock protein 70 kDa family member 13 precursor	0.2
NP_057383	Heat shock 70-kDa protein 14 isoform 1	Detected only in glaucoma
XP_497902	Similar to heat shock cognate 71-kDa protein	Detected only in glaucoma
XP_496273	Similar to mitochondrial precursor 75-kDa glucose regulated protein	2.0*
NP_004125	Heat shock 70kDa protein 9; 75-kDa glucose regulated protein	4.6*
NP_057376	Heat shock protein 75	1.9*
NP_006810	Stress-induced phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	Detected only in glaucoma
XP_496420	Similar to heat shock protein HSP90-alpha (HSP86)	0.2
XP_374273	Similar to heat shock protein HSP90-beta (HSP84)	0.3
NP_005339	Heat shock 90-kDa protein 1, alpha	3.6*
NP_001035231	Heat shock 90-kDa protein 1, alpha-like 3	Detected only in glaucoma
NP_031381	Heat shock 90-kDa protein 1, beta	4.9*

Individual retinal protein samples obtained from human donor eyes with ($n = 10$) or without ($n = 10$) glaucoma were used in quantitative LC-MS/MS analysis. 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. Note that some of the identified proteins were detectable only in glaucomatous samples. RefSeq is provided in the public domain by the National Center for Biotechnology Information. Available at www.ncbi.nlm.nih.gov/locuslink/refseq/.

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

Glial cells exposed to HSPs or oxidative stress were then co-cultured with T cells, to determine alterations in their antigen-presenting ability. After a 48-hour co-culture, we detected a prominent stimulation of glial antigen presentation as assessed by increased proliferation of co-cultured T cells (Fig. 5). Co-culturing T cells with the microglia or astrocytes pretreated with HSPs or H_2O_2 resulted in approximately fourfold and twofold increases in T-cell proliferation, respectively. Treatment of microglia with MyD88- or TIRAP-inhibiting treatments resulted in a significant decrease in the stimulated T-cell proliferation (Fig. 5A; Mann-Whitney rank sum test, $P < 0.01$). However, similar treatment of astrocytes did not result in a significant alteration in the stimulation of co-cultured T cells (Mann-Whitney rank sum test, $P > 0.05$), except for a significant effect of the MyD88 inhibitor in co-cultures with the astrocytes exposed to HSP60 (Fig. 5B; Mann-Whitney rank sum test, $P < 0.01$).

TNF-α is the cytokine that is most abundantly produced by T cells during glial antigen presentation.[16,36,39] Control experiments in glial cells pretreated with LPS resulted in a more than fivefold increase in TNF-α secretion, as well as proliferation, of co-cultured T cells; and this stimulation was significantly reduced by glial MyD88- or TIRAP-inhibiting treatments (Mann-Whitney rank sum test, $P < 0.01$). The TNF-α levels measured by ELISA were also significantly higher when the T cells were co-cultured with the microglia (more than fivefold) or astrocytes (more than threefold) pretreated with HSPs or H_2O_2 (Fig. 5). The pretreatment of glial cells with mitomycin C before mixing them with T cells excluded the possibility that this cytokine was produced by glial cells in co-cultures. The stimulation of T-cell cytokine production in co-cultures with microglia was significantly decreased by specific treatments inhibiting MyD88 or TIRAP (Fig. 5C; Mann-Whitney rank sum test, $P < 0.01$). However, no statistically significant treatment effect was detected in the T-cell cytokine secretion in co-cultures with astrocytes (Mann-Whitney rank sum test, $P > 0.05$), with the exception of the MyD88-inhhibiting treatment of HSP60-exposed astrocytes (Fig. 5D; Mann-Whitney rank sum test, $P < 0.01$).

DISCUSSION

Upregulation of TLRs in the Glaucomatous Human Retina

Findings of this study, based on proteomic and immunohistochemical analyses, consistently showed an upregulation of some TLRs in the glaucomatous human retina. Immunolabeling of donor tissue sections with specific antibodies demonstrated localization of TLR2, -3, and -4 on retinal microglia and astrocytes.

One of the mechanisms by which the innate immune system senses the invasion of pathogenic microorganisms is through the TLRs, which recognize specific molecular patterns present in microbial components. After engaging the pathogenic patterned ligands, the cytosolic portion of the

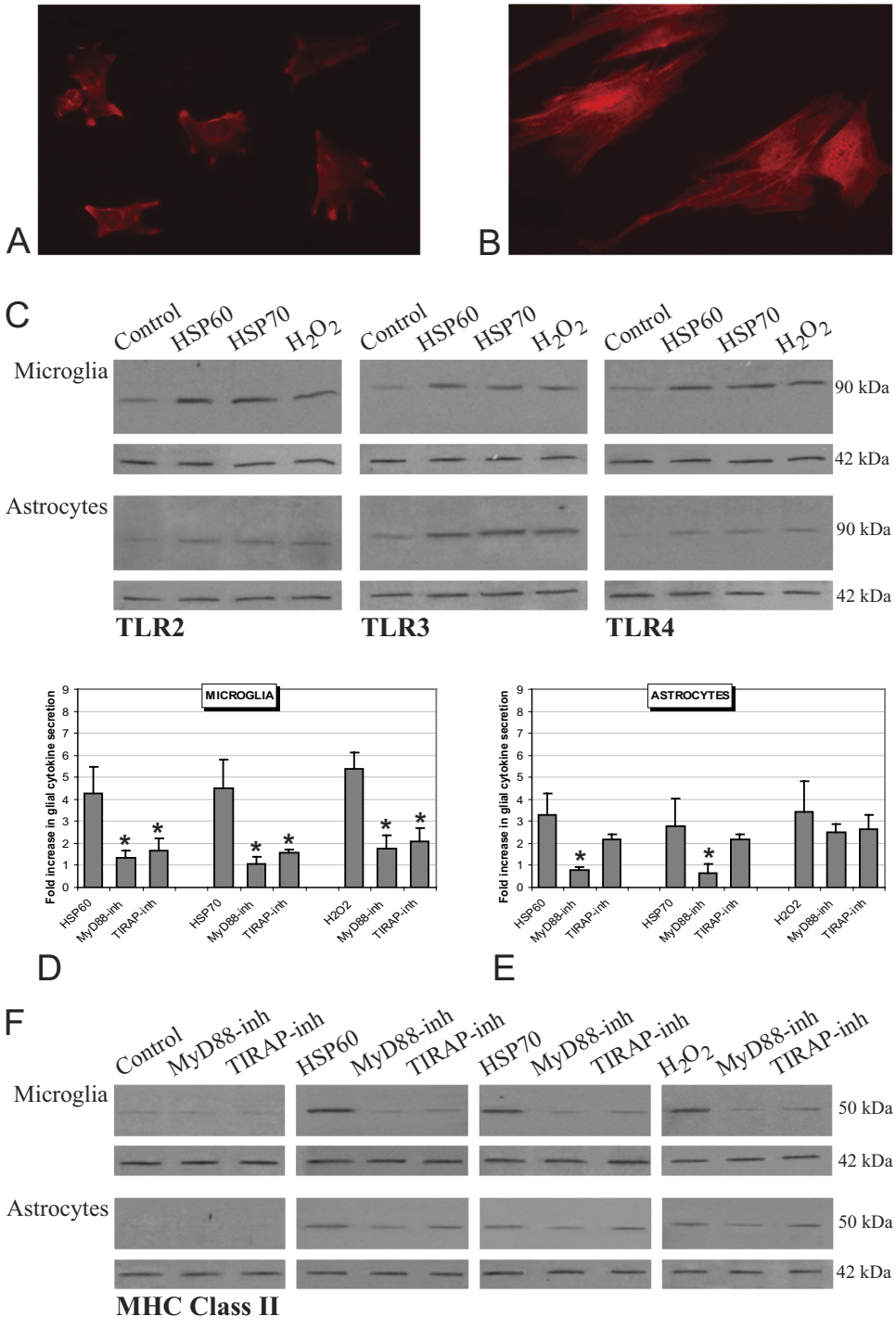

FIGURE 4. In vitro experiments determining glial TLR signaling. Cell cultures of microglia and astrocytes were derived from rat retinas. (**A**) Fluorescence images of microglial cells immunolabeled for CD11b and (**B**) of astrocytes immunolabeled for GFAP. (**C**) Western blot analysis detected TLR expression in protein samples obtained from cultured microglia and astrocytes. Whereas the expression of TLR2, -3, and -4 was prominently detectable in the microglia, the expression of TLR3 was predominant in the astrocytes. After normalization to β-actin (42-kDa bands), the average band intensity obtained from untreated samples was used to calculate the ratios of change in protein expression in the treated samples. Based on the normalized intensity values, glial TLR expression exhibited a more than twofold upregulation after exposure to HSPs or H_2O_2 (Mann-Whitney rank sum test; $P < 0.01$). (**D**, **E**) The TNF-α production of these glial cells also exhibited a significant increase after exposure to HSPs or H_2O_2. Treatment of glial cells to inhibit MyD88 and TIRAP, the adaptor proteins of TLR signaling, resulted in a significant decrease in HSPs or H_2O_2-induced cytokine production of the microglia. Regarding astrocytes, only MyD88-inhibiting treatment resulted in a significant decrease in cytokine production of HSP60- or HSP70-treated astrocytes. *Statistical significance (Mann-Whitney rank sum test; $P < 0.01$). Cytokine production was assessed by ELISA, and the data are presented as the mean change ratio (±SD), which was determined by comparing the normalized values in treated cultures with those in untreated control cultures. (**F**) Exposure to HSPs or H_2O_2 upregulated glial MHC class II expression. Based on the β-actin-normalized intensity levels, treatment with MyD88 and TIRAP inhibitors resulted in a significant decrease in the microglial MHC class II upregulation (Mann-Whitney rank sum test; $P < 0.01$). However, only MyD88-inhibiting treatment provided a significant treatment effect in astrocytes (Mann-Whitney rank sum test; $P < 0.01$). All experiments were performed in triplicate wells and independently repeated three times for each experimental condition.

TLRs recruits adaptor proteins and activates the transcription factor nuclear factor-kappaB (NF-κB), thereby leading to the expression of proinflammatory cytokines. Such rapid, innate cellular responses serve as the first line of host defense mechanisms and also stimulate antigen-specific adaptive immunity to eliminate pathogens and facilitate tissue healing. The emergence of an additional role for TLRs in sensing nonpathogenic stimuli has expanded their repertoire, so that these receptors are now also considered to be innate immune surveillance receptors for danger signals independent of infection.[17,18] In addition to important roles of TLRs in innate[40] and T-cell-mediated immune activities[40,41] in sensing intrinsic stimuli, recent studies of mice deficient in TLRs also support their roles in autoantibody-mediated immunity in different disease models.[42,43] TLRs have been studied in the retina,[44,45] and various major roles have been proposed in the regulation of innate and adaptive immune responses linked to aging-related macular degeneration[46] and retinal progenitor cell proliferation.[47] The upregulated expression of glial TLRs detected in the glaucomatous human retina, along with the in vitro observations of a TLR-mediated increase in glial cytokine production and antigen presentation, also support an association of TLRs with the modulation of immune response in glaucoma.

We used a label-free LC-MS/MS approach for quantitative proteomic analysis of human retinal protein samples. Because

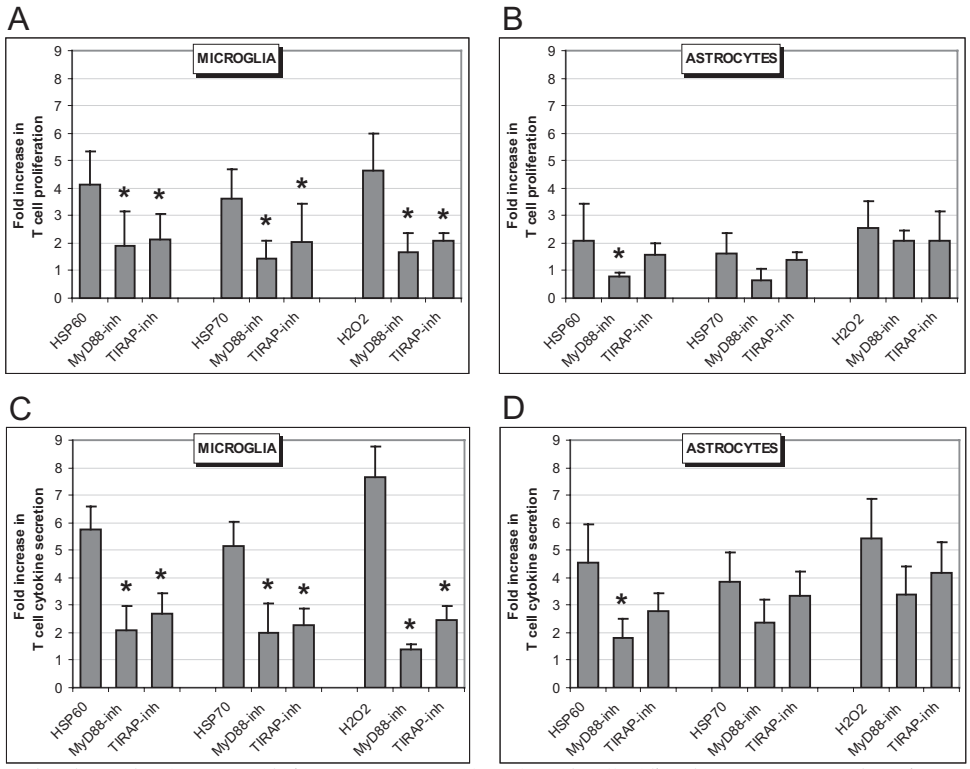

FIGURE 5. In vitro experiments determining glial antigen presentation. Alterations in glial antigen-presenting ability were determined by co-culturing retinal microglial cells and astrocytes pretreated with HSPs or H_2O_2 with T cells and measuring T-cell proliferation and TNF-α secretion at 48 hours. Exposure of microglial cells (**A**, **C**) and astrocytes (**B**, **D**) to exogenous HSP60 or -70 or to H_2O_2-induced oxidative stress resulted in increased proliferation and cytokine secretion of co-cultured T cells. After treatments inhibiting MyD88 and TIRAP, the adaptor proteins of TLR signaling, microglial antigen presentation decreased significantly. *Statistical significance (Mann-Whitney rank sum test; $P < 0.01$). However, MyD88-inhibiting treatment resulted in a significant decrease in T-cell proliferation and cytokine secretion only in co-cultures with astrocytes exposed to HSP60, whereas TIRAP-inhibiting treatment did not significantly affect the stimulated antigen presentation by astrocytes (Mann-Whitney rank sum test; $P > 0.05$). All experiments were performed in triplicate wells and independently repeated three times in newly cultured cells. The data are presented as the mean ratio of change (±SD), which was determined by comparing the normalized values in treated cultures with those in untreated control cultures.

of the complexity of sample processing and data interpretation and the substantial cost associated with using stable-isotope methods, label-free methods have become more widely used.[48,49] A primary advantage of chemical labeling is that comparative samples are mixed before mass spectrometric analysis, thereby eliminating the experimental variability caused by sample preparation and analysis. However, a previous studies using the label-free approach have demonstrated a high level of reproducibility across LC-MS/MS experiments,[28] and many of the label-free quantitative differences between disease versus control samples have been validated by immunoblot analysis and immunohistochemistry.[22,25]

It should also be clarified that despite their unique importance, studies of human donor tissues may be challenging because of the retrospective nature of data collection, the difficulties in excluding other disease conditions or treatment effects, and the possibility of perimortem tissue alteration. However, we were careful to minimize such confounding factors, and the tissues from glaucomatous and nonglaucomatous donors were matched for the donor age, cause of death, and postmortem period. In addition, proteomic and immunohistochemical findings in the human tissues were supplemented and further explored in in vitro experiments.

Glaucomatous Stress-Related Ligands of TLRs That Mediate Glial Immunoregulatory Functions

Findings in the in vitro experiments supported that HSPs and oxidative stress, widely recognized to be generated by glaucomatous tissue stress, may exert immunoregulatory functions via glial TLRs. Through the TLR signaling, such intrinsic ligands can serve as danger signals and stimulate innate and adaptive immunity as evident by increased cytokine production and MHC class II expression of glial cells and increased proliferation and cytokine production of co-cultured T cells.

In addition to increasing the stress resistance, HSPs have been recognized as immunodominant antigens and implicated as targets of antigen-specific immune activity in various autoimmune diseases.[50,51] Self HSP-specific T and B cells have been found in association with different disease conditions, as well as in healthy individuals.[52,53] Similarly, HSPs seem to be upregulated targets of immune activity in glaucoma as supported by previous studies demonstrating increased titers of HSP antibodies in the glaucomatous patient's serum.[11–13] Although the immune response to upregulated HSPs in glaucoma may reflect normal immune surveillance, growing evidence over the past decade supports that HSPs can actively signal tissue destruction and danger to the immune system. Extracellular HSPs after active secretion or passive release from dying cells, as well as membrane-bound HSPs, may bind to the surface of adjacent cells and initiate signal transduction cascades, thereby expanding the stress response into the extracellular microenvironment. There is evidence supporting immunomodulatory features of HSPs both at the level of innate defense mechanisms and at the level of antigen-specific adaptive immunity.[17,54] For example, proinflammatory signaling cascades induced by HSPs may lead to the activation of antigen-presenting cells. HSPs are also able to transport antigenic peptides and deliver them to MHC class I and II molecules on antigen-presenting cells by even acting at distant sites in the body after entering the blood circulation and transporting intracellular peptides to distant immune cells.[55,56] A range of powerful immune effects of HSPs are transmitted by several cell surface receptors including TLRs.[17,18,57] Better understanding of endogenous molecular pathways has made it clear that the TLR signaling activated by HSPs mediates neuroinflammation and neurodegeneration in the central nervous system.[19,20] However, it should be emphasized that although TLRs are now widely considered to be HSP receptors, interactions between TLRs and HSPs may not be exerted through the direct binding of HSPs, but may be

through an indirect mechanism involving HSP binding to a primary receptor that secondarily activates TLR signaling.

Our in vitro findings suggest that the HSPs upregulated in glaucoma may similarly be associated with the initiation of immune response through the glial TLR signaling. Of interest, autoantibodies against HSPs have also been proposed to play a pathogenic role in autoimmune diseases by enhancing inflammatory reactions and cytokine production via TLRs.[58] We wonder whether increased HSP antibodies in the glaucomatous patient sera may have similar effects through TLRs. It is also tempting to determine whether the immunogenic activity leading to RGC injury after immunization of rats with HSPs[6] has links to the TLR signaling. An important aspect of the HSP-mediated immunity has been associated with the molecular mimicry. For example, various autoimmune diseases can be triggered by cross-reactive T cells that recognize common epitopes in mammalian and highly immunogenic prokaryotic HSPs.[50,59] This association brings up another interesting question as to whether infectious factors affect immune system regulation through TLR signaling in glaucoma.

Experimental evidence of oxidative stress due to mitochondrial dysfunction[37] along with the age-related component of oxidative stress in glaucoma[21] have long been linked with various cellular events contributing to the neurodegenerative injury,[60] which include immunostimulation.[3] In an in vitro study, we found that oxidative stress stimulates antigen presentation by retinal glial cells.[16] New in vitro findings provide additional evidence that the regulation of glial immune functions by oxidative stress is mediated through TLR signaling. There is much evidence that provides mechanistic links between oxidative stress and immune activity through TLRs.[61] For example, oxidized phospholipids have been shown to modulate TLR activation.[62–64] Oxidative stress–induced immune activity has been linked to TLRs in different disease models,[65,66] in which oxidant-TLR interactions have been shown to provide a signal that initiates inflammatory processes.[67,68] TLR signaling has been found to increase the vulnerability of neurons to oxidative stress in Alzheimer's disease,[69] whereas TLR deficiency has been found to confer susceptibility to oxidative injury in other tissues.[70,71] The precise mechanisms by which oxidants mediate these effects are unclear, but could be due to oxidative protein modifications. For example, oxidation of the extracellular portion of the receptor may cause a conformational change or redox modifications of specific signaling molecules may result in functional alterations. Our previous observations of oxidative modification of many retinal proteins in experimental glaucoma[23] may support the likelihood of this suggestion. Of interest, in our previous study,[23] we identified HSPs among oxidatively modified retinal proteins during glaucomatous neurodegeneration. This piece of evidence motivates additional research to determine whether oxidative modifications may further increase the immunostimulatory features of HSPs in glaucoma. As implied by results in other disease models,[72] another oxidative stress–related intrinsic ligand for TLR signaling in glaucoma could be advanced glycation end products, the production of which is also increased in human glaucoma.[21]

TLR Signaling in Glaucoma

Bioinformatics analysis of our mass spectrometric data obtained from human retinal protein samples highlighted numerous molecules involved in the TLR signaling pathway that result in diverse immune responses. Our in vitro experiments with HSPs and H_2O_2-induced oxidative stress demonstrated that these glaucomatous stress-related ligands can activate glial TLRs and stimulate the antigen-presenting function of glial cells through the TLR signaling.

Thirteen different TLRs have been described so far that recognize distinct classes of ligands resulting in innate and adaptive immune responses. Recent accumulating evidence supports that Toll/interleukin (IL)-1-receptor (TIR) domain–containing adaptors, such as MyD88 (myeloid differentiation primary-response protein 88), TIRAP (TIR-domain–containing adaptor protein, also known as Mal), TRIF (TIR-domain–containing adaptor protein inducing IFN-β, also known as TICAM-1), and TRAM (TRIF-related adaptor molecule), modulate the TLR signaling. MyD88 is the immediate adaptor molecule essential for the induction of inflammatory cytokines, which is common to all TLRs, with the exception of TLR3. The MyD88-dependent pathway activates IRAKs (IL-1-receptor-associated kinases) and TRAF6 (TNF-receptor-associated factor 6), and leads to the activation of the IKK (inhibitor of NF-κB-kinase) complex. This pathway is used by TLR1, -2, -4, -5, -6, -7, and -9 and releases NF-κB from its inhibitor so that it translocates to the nucleus and induces the expression of inflammatory cytokines.[73–75] TIRAP is specifically involved in the MyD88-dependent pathway via TLR2 and -4, whereas TRIF is implicated in the TLR3- and -4-mediated MyD88-independent pathway. A fourth TIR-domain–containing adaptor, TRAM, is specific to the TLR4-mediated, MyD88-independent/TRIF-dependent pathway. Thus, TIR domain–containing adaptors provide the specificity of TLR signaling.[76] Of interest, however, there is also evidence supporting cross-regulation between the different TLRs.[77] Findings of our in vitro experiments using specific treatments suggest that the TLR signaling initiated by HSPs or oxidative stress includes MyD88- and TIRAP-dependent pathways in the retinal microglia. However, it seems more likely that MyD88- and TIRAP-independent pathways are predominant in the TLR signaling triggered in retinal astrocytes. The differential signaling pathways observed in retinal microglia and astrocytes may be explained by the predominant expression of TLR3 in astrocytes, as opposed to a similar expression pattern of TLR2, -3, and -4 in microglia. However, further study is needed to determine the specific signaling components involved in the glial TLR signaling initiated by stress-related ligands.

TLR signaling is also known to be modulated by several endogenous inhibitors at various points in the downstream pathways for fine tuning, and if there is a defect in these regulatory mechanisms, systemic autoimmunity will result rather than immunoregulation. Getting the correct balance between activation and inhibition is therefore critical for ensuring that TLRs do their immunoregulatory job properly without leading to immunopathology.[78]

In summary, findings of this study support that danger signals to the immune system may be transmitted by HSPs and oxidative stress-related products through the glial TLR signaling. These findings warrant additional in vitro and in vivo studies using transgenic mouse models so that the importance and molecular mechanisms of glial TLR signaling through different receptor subtypes can be further clarified. An improved understanding of how tissue stress, neuronal injury, and glial response during glaucomatous neurodegeneration orchestrate individual components of immunity via TLRs can help in the design of immunomodulation-based treatment strategies.

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