Iris Pigment Epithelial Cells Express a Functional Lipopolysaccharide Receptor Complex

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Purpose. Ocular pigment epithelial cells are hypothesized to play a role in the pathogenesis of acute anterior uveitis (AAU), where LPS activation of Toll-like receptors (TLRs) may serve as a trigger. In this study, the expression of LPS receptors in iris pigment epithelium (IPE) was determined.

Methods. RT-PCR, flow cytometry, Western blot, and immunohistochemistry were used to investigate the expression of the LPS receptor complex (TLR4, MD-2, and CD14) in primary human IPE. Cytokine secretion by LPS-treated IPE was measured by multiplex bead array and ELISA. The role of CD14 in modulating the LPS response was investigated by addition of soluble CD14 and by antibody neutralization studies. In vivo expression of CD14 was examined by immunohistochemistry and Western blot analysis.

Results. IPE expressed TLR4, MD-2, and CD14 in vitro and secreted a panel of proinflammatory cytokines (IL-6, CXCL8, CXCL10, CCL2, CCL4, and CCL5) when stimulated with LPS. CXCL8 secretion by LPS-treated IPE was dependent on CD14 and TLR4. CD14 was detected in CD68+ cells in the iris by immunohistochemistry and in normal aqueous by Western blot analysis.

Conclusions. IPE cells express a functional LPS receptor complex and are capable of promoting ocular inflammation through secretion of an array of proinflammatory mediators. CD14 was identified as a key molecule that modulated the LPS response in IPE. (*Invest Ophthalmol Vis Sci.* 2010;51: 2558–2567) DOI:10.1167/iovs.09-3923

Uveitis comprises a heterogeneous group of intraocular inflammatory diseases that affect the uveal tract of the eye. It is characterized by breakdown of the blood–ocular barrier and infiltration of the uvea with inflammatory cells mediated by locally produced cytokines and chemokines.[1,2] Uveitis may involve any part of the uvea, but the commonest form is acute anterior uveitis (AAU) affecting the iris and ciliary body and accounting for up to 90% of all cases. The condition occurs predominantly in individuals of working age and may result in significant visual impairment due to its recurrent or chronic nature.[3,4]

The pathogenesis of AAU is poorly understood, although genetics[3] and T-cell-mediated autoimmunity[5–7] are recognized to play a role in its development. More recently, lipopolysaccharide (LPS) activation of innate immune receptors such as Toll-like receptors (TLRs) has been hypothesized to play a role in AAU.[8] Supporting this idea are animal models of endotoxin-induced uveitis (EIU)[9] and clinical observations in humans, where AAU is temporally associated with Gram-negative infections of the gastrointestinal[10] or urogenital tract.[11] In patients with AAU, the presence of antibodies to microbial antigens is associated with recurrent disease.[12] Furthermore, TLR4 hyporesponsiveness and downregulation of TLR2 expression in the peripheral blood monocytes and neutrophils of these patients suggests endotoxin tolerance.[13]

A trimolecular complex comprising TLR4, MD-2 (myeloid differentiation protein-2), and CD14 is necessary to detect LPS.[14–16] TLR4 is the principle signaling molecule but does not interact directly with LPS. Instead, this role is performed by the co-receptors MD-2 and CD14. MD-2 is a soluble protein that forms a stable complex with TLR4. LPS interacts with MD-2 leading to conformational changes that trigger TLR4 signaling.[17,18] CD14 is a glycosylphosphatidylinositol-anchored membrane protein (mCD14) that also exists in a soluble form (sCD14), and it binds and presents LPS to the TLR4/MD-2 complex.[19] mCD14 is preferentially expressed by cells of myeloid lineage, but may also be expressed by nonmyeloid cells.[20] sCD14 in the extracellular fluid replaces the function of mCD14 in cells that lack this glycoprotein.[21] Components of the LPS receptor complex have been studied in human uveal tissue. We have reported the co-localization of TLR4, MD-2, and CD14 in HLA-DR$^+$ dendritic cells within the iris stroma.[22] Others have reported TLR4 and CD14 expression by nonpigmented ciliary body epithelium (CBE),[23] retinal pigment epithelium (RPE),[24,25] and TLR4 in cultured iris endothelial cells.[23] sCD14 is present in tears[26] and serum,[27] and serum CD14 may become elevated in inflammatory diseases such as rheumatoid or reactive arthritis[27] and systemic lupus erythematosus[28] and during sepsis[29] when CD14 production maybe upregulated by LPS.[30] Although not previously described, it is conceivable that sCD14 is elevated locally or systemically during active uveitis. Huhtinen et al.[31] suggested that expression of mCD14 in the peripheral blood monocytes of patients with inactive AAU is no different from that in control subjects. It was noted however, that monocytes from these patients showed increased TNF-α production to LPS ex vivo, implying high innate immune responsiveness. We speculate that ele-

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TABLE 1. Primer Sequences and PCR Conditions

Gene	Primer Pair	Annealing Temperature (°C)	Cycles	Product Size (bp)
TLR2	F: 5′-GTACCTGTGGGGCTCATTGT-3′ R: 5′-CTGCCCTTGCAGATACCATT-3′	62	35	191
TLR4	F: 5′-TACAAAATCCCCGACAACCTC-3′ R: 5′-AGCCACCAGCTTCTGTAAACT-3′	60	35	264
MD-2	F: 5′-GAAGCTCAGAAGCAGTATTGGGTC-3′ R: 5′-GGTTGGTGTAGGATGACAAACTCC-3′	62	28	422
CD14	F: 5′-AGAGGCAGCCGAAGAGTTCAC-3′ R: 5′-GCGCTCCATGGTCGATAAGT-3v	60	35	132
GAPDH	F: 5′-ACCACAGTCCATGCCATCAC-3v R: 5′-TCCACCACCCTGTTGCTGTA-3′	60	28	452

F, forward primer; R, reverse primer.

vated sCD14 (which was not examined) explains their observations.

IPE and RPE share embryologic origins from neuroectoderm,[32,33] and there is evidence to suggest that IPE transplantation substitutes for RPE in diseases such as atrophic age-related macular degeneration.[34–36] Both cell types contribute to immune privilege by producing transforming growth factor-β, somatostatin, thrombospondin, and pigment epithelium–derived factor and promoting development of regulatory T cells.[37,38] However, their expression of cytokine transcripts[39] implies that they may play a proinflammatory role.

In this study, the role of IPE in the pathogenesis of AAU was investigated. We explored their expression of the LPS receptor complex and cytokine secretion in response to LPS stimulation, using the well-characterized ARPE-19 cells as the control. We showed, for the first time, that IPE expresses a functional LPS receptor complex, and secretes a panel of proinflammatory mediators when stimulated with LPS. Furthermore, neutralization experiments suggest that the LPS response of IPE is dependent on CD14 and TLR4.

METHODS

Ocular Tissues and Aqueous Samples

Human eyes ($n = 3$) obtained from the Lions Eye Bank (Sydney, Australia) were subjected to formalin fixation and paraffin embedding. Normal aqueous was collected from noninflamed eyes of patients undergoing cataract surgery at St. Vincent's hospital (Sydney) after informed consent was obtained from each subject. Aqueous was stored at −80°C until needed. Collection and management of all human samples adhered to the tenants of the Declaration of Helsinki and had institutional human ethics committee approval.

Cell Cultures and Reagents

Primary human IPE and epithelial cell medium (EpiCM) supplemented with 2% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and epithelial cell growth supplement (content not disclosed by the manufacturer), were purchased from ScienCell Research Laboratories (San Diego, CA). For controls, the spontaneously arising retinal pigment epithelial cell line (ARPE-19) characterized by Dunn et al.,[40] was kindly provided by the Lions Eye Bank. The ARPE-19 cells were cultured in a 1:1 mixture of DMEM and Ham's-F12 medium supplemented with 2% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Primary IPE (passages 2–3) and ARPE-19 (passage 14–20) were used in the experiments.

LPS from *Escherichia coli* (serotype O55:B4, Sigma-Aldrich, St. Louis, MO) was dissolved in PBS and stored in 1 mg/mL aliquots at −20°C. For stimulation experiments, LPS was diluted to final concentration in serum-free medium (SFM). Carrier-free recombinant human CD14 (CHO cell derived, <0.1 ng endotoxin/μg CD14), anti-human CD14 (clone 134620, neutralizing), and isotype IgG_1 (clone 11711) were purchased from R&D Systems, Inc. (Minneapolis, MN). Neutralizing antibodies against TLR2 (clone TL2.1) and TLR4 (clone HTA125) and isotype control antibody (IgG2a, κ) were purchased from eBio-

TABLE 2. Primary Antibodies Used for Immunohistochemistry, Western Blot Analysis, and Flow Cytometry

Antigen Specificity	Antibody Subtype	Label	Clone	Manufacturer	Working Concentration or Dilution
CD14*	Mouse IgG_{2a}	—	7	Novacastra	1:100
CD68*	Mouse IgG_3	—	PG-M1	Dako	1:200
CD207*	Rat IgG_{2a}	—	DDX0362	Dendritics	1:100
Isotype*	Mouse IgG_{2a}	—	—	Dako	1:100
TLR4†	Goat IgG	Biotin	—	R&D Systems	0.2 μg/mL
CD14†	Mouse IgG_1	—	134620	R&D Systems	2 μg/mL
GAPDH†	Mouse IgG_1	—	ID4	Imgenex	1 μg/mL
TLR2‡	Mouse IgG_{2a},κ	FITC	TL2.1	Imgenex	20 μg/mL
TLR4/MD-2 complex‡	Mouse IgG_{2a},κ	PE	HTA125	eBioscience	20 μg/mL
CD14‡§	Mouse IgG_{2b}	PerCP	MΦP9	BD Biosciences	10 μg/mL
Isotype‡	Mouse IgG_{2a},κ	FITC	—	Dako	20 μg/mL
Isotype‡	Mouse IgG_{2a},κ	PE	—	eBioscience	20 μg/mL

Novacastra, Newcastle-upon-Tyne, UK; Dako, Carpinteria, CA; Dendritics, Lyon, France; R&D Systems, Minneapolis, MN; Imgenex San Diego, CA; eBioscience, San Diego, CA; BD Biosciences, San Diego, CA.

* Antibodies used for immunohistochemistry.

† Antibodies used for Western blots.

‡ Antibodies used for flow cytometry.

§ Isotype control was unavailable for this antibody.

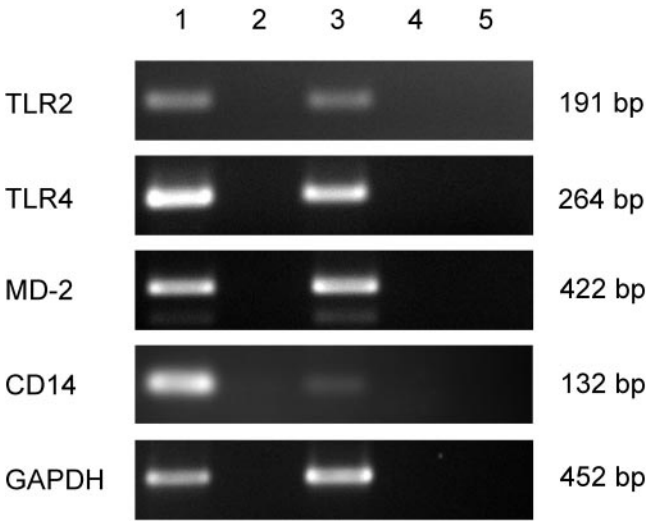

FIGURE 1. TLR and co-receptor transcripts in ocular pigment epithelial cells. One microgram of total RNA from IPE and ARPE-19 cells was reverse transcribed and used as the template for PCR. Amplicons for TLR2, TLR4, MD-2, CD14, and GAPDH were generated and visualized on an ethidium bromide–stained agarose gel. Specific PCR products were detected in cDNA from IPE (*lane 1*) and ARPE-19 (*lane 3*), but not in their corresponding RT negative controls (*lane 2*, IPE; *lane 4*, ARPE-19) nor in the no-template control (*lane 5*).

science, Inc. (San Diego, CA). Unless otherwise stated, all cell culture reagents were from Invitrogen (Carlsbad, CA).

Semiquantitative RT-PCR Detection of LPS Receptor Complex

Total RNA was isolated from IPE and ARPE-19 cultures with a total RNA isolation system (RNAgents; Promega Corp., Madison, WI). One microgram of RNA was reverse transcribed into cDNA (SuperScript III RT system; Invitrogen) with oligo dT primers in a 20-μL reaction, and PCR was performed (GeneAmp PCR system 2400; Perkin Elmer, Boston MA). Each reaction consists of 1 μL of cDNA, 200 nM each of sense and antisense primers (Table 1), 200 μM dNTPs, 2.5 mM MgCl, 1 U DNA polymerase (Platinum *Taq*; Invitrogen) and reaction-grade water to 20 μL. PCR conditions were as follows: initial incubation at 95°C for 2 minutes, followed by three-step cycling (denaturing at 95°C for 30 seconds, annealing temperature for 30 seconds [Table 1], extension at 72°C for 30 seconds), and a final extension step at 72°C for 2 minutes. PCR products were displayed on an ethidium bromide–stained 2.5% wt/vol agarose gel.

Western Blot Detection of CD14 and TLR4

Whole-cell lysates were prepared by incubating cells in ice cold lysis buffer (0.1% SDS, 0.5% NP-40 in 50 mM Tris-HCl [pH 7.4]) supplemented with a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail; Roche, Mannheim, Germany). After 30 minutes, cell lysates were centrifuged at 10,000*g* for 10 minutes at 4°C and protein concentration of supernatants were determined by a modified Lowry method (DC protein assay; Bio-Rad, Hercules, CA). Cell lysates (20 μg) and normal aqueous from patients undergoing cataract surgery (10–20 μL) were separated by 10% SDS-PAGE and transferred onto PVDF (NEF1002; Perkin Elmer). Membranes were blocked overnight in 5% skim milk/TBST (pH 7.6) at 4°C, followed by incubation with primary antibodies (Table 2) in 2% BSA/TBST for 1 hour at room temperature. After they were washed in TBST, the membranes were incubated with either HRP-conjugated goat anti-mouse IgG (1:2000 dilution) or HRP-conjugated streptavidin (1:1000 dilution) for 1 hour at room temperature (both from Dako, Carpinteria, CA). Finally, membranes were washed three times in TBST and developed with enhanced chemiluminescent Western blot analysis substrate (Pierce-Thermo Fisher Scientific Inc., Rockford, IL).

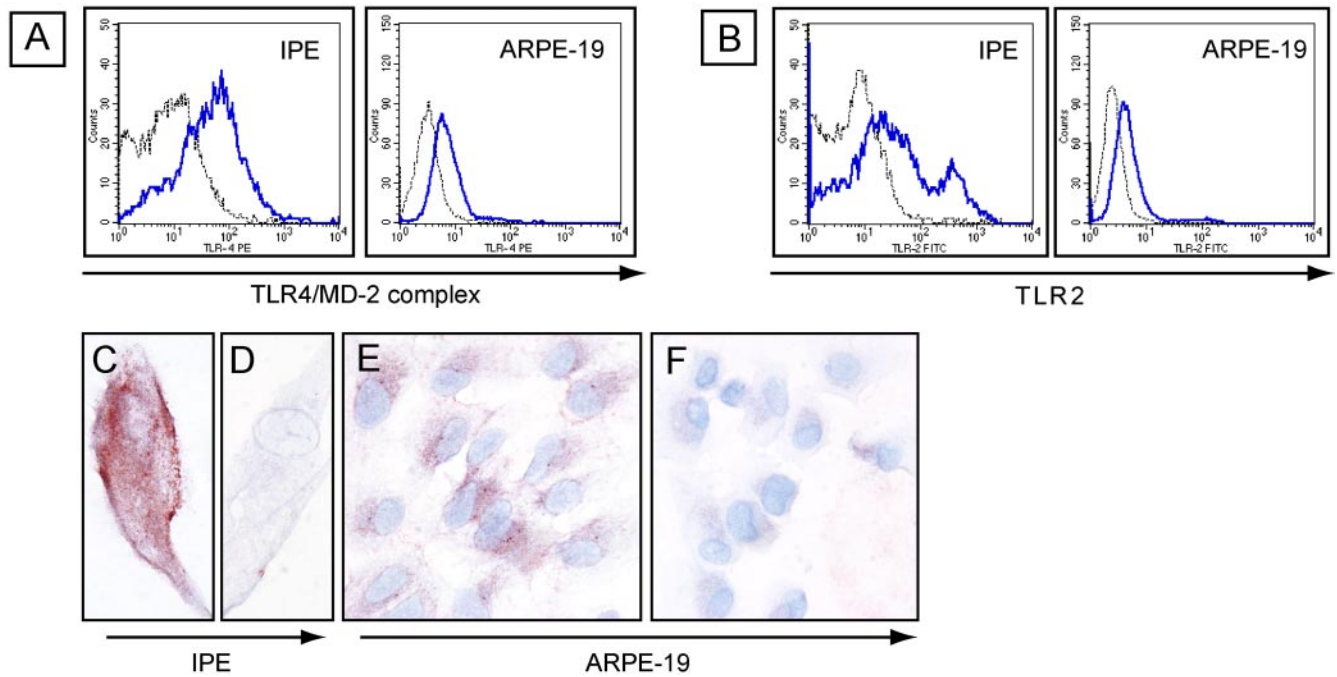

FIGURE 2. Expression of TLR2, TLR4/MD-2, and CD14 in ocular pigment epithelial cell cultures. IPE and ARPE-19 expressed TLR4/MD-2 (**A**) and TLR2 (**B**), as indicated by flow cytometry. *Solid lines*: specific labeling by anti-TLR4/MD-2 complex antibody (clone HTA125), anti-TLR2 (clone TL2.1); *dashed lines*: nonspecific labeling by isotype control (IgG2a). CD14-immunoreactivity (*red*) was identified in cultured IPE (**C**, **D**) and ARPE-19 (**E**, **F**) cells, but only in cells incubated with rabbit anti-CD14 (**C**, **E**). Staining was not evident in cells incubated with isotype IgG (**F**) or in the absence of a primary antibody (**D**). (**C**–**F**) Original magnification, ×1000.

Flow Cytometry Analysis

Flow cytometry was performed with modifications to a previous protocol.[41,42] Briefly, IPE and ARPE-19 cells were cultured in 25 cm^2 culture flasks until they reached subconfluence. The cells were detached with 0.05% trypsin/0.02% EDTA solution, and the enzyme mixture inactivated with complete medium (for ARPE-19) or trypsin-neutralizing solution (for IPE, content not disclosed by ScienCell). The cells were washed once in PBS, left to recover in SFM for 1 hour in a humidified cell culture incubator set to 37°C and 5% CO_2, and incubated with antibodies to TLR2, TLR4/MD-2 complex, CD14 or corresponding isotype control (Table 2) for 30 minutes on ice. Immunolabeled cells were washed in 1% BSA in PBS and resuspended in 300 μL of 1% paraformaldehyde in PBS. Data were acquired by flow cytometry (LSR II Flow Cytometer; BD Biosciences, San Jose, CA), and the results analyzed (CellQuest Pro software; BD Biosciences).

Immunohistochemical Analysis of Ocular Tissue and in Cultured Pigment Epithelial Cells

Four-micrometer sections of ocular tissues were dewaxed in xylene and rehydrated through a graded series of ethanols. Antigen retrieval was performed by boiling tissues for 10 minutes in 0.01 M citrate buffer (pH 6.0). The sections were blocked for 20 minutes in serum-free protein block (X0909; Dako, Glostrup, Denmark), followed by incubation in primary antibodies (Table 2) for 16 hours at 4°C. After TBS washes, the sections were next incubated in 10 μg/mL of Alexa Fluor 488- or 546-conjugated secondary antibodies (A-11001, A-21208 or A-11003; Invitrogen) for 30 minutes at room temperature. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and the sections mounted (Vectashield; Vector Laboratories, Burlingame, CA).

IPE and ARPE-19 cells were cultured on poly-L-lysine–coated, four-well slides (CultureSlides, 354114; BD Biosciences). For immunolabeling of CD14, the cells were washed with PBS, fixed in acetone for 5 minutes at room temperature, and air dried. After the cells were rehydrated in TBS, they were blocked in goat serum before incubation in rabbit anti-CD14 (2 μg/mL, sc-9150; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 16 hours. The cells were subsequently washed in TBS and incubated in biotinylated goat anti-rabbit IgG (Dako) at 1:200 dilution for 30 minutes at room temperature, followed by a 1-hour incubation in streptavidin-HRP at 1:100 (Dako). Specific immunoreactivity was visualized by 3-amino-9-ethylcarbazole chromogen (AEC). Photomicrographs were captured with a microscope (BX51; Olympus, Tokyo, Japan) attached to a DP70 digital camera.

Bead-Based Multiplex Array Analysis of LPS-Stimulated Cell Cultures

IPE and ARPE-19 cells were seeded at 5000 cells/cm^2 into 24-well plates and cultured until 90% confluent. After two washes with PBS, the cells were exposed to 0 to 10 μg/mL of LPS in SFM (500 μL per well) at 37°C in a 5% CO_2 incubator. Culture supernatants were collected at 24 hours, spun down to remove cell debris, and stored at −80°C until analysis. A 15-plex bead array targeting IL-1β, IL-6, IL-10, IL-12, IL-17, TNF-α, IFN-γ, CXCL8 (IL-8), CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP1–1β), CCL5 (RANTES), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF; Bio-Rad), was used to detect cytokines, chemokines, and growth factors from cell culture supernatants.

CD14 Experiments and Enzyme-Linked Immunosorbent Assay

The role of CD14 in modulating the LPS response in cell cultures was investigated by addition of recombinant CD14 and by antibody inhibition studies. IPE and ARPE-19 were seeded into 24-well plates and cultured until 90% confluent. The cells were washed twice with PBS and serum starved for 18 hours before treatment with carrier-free recombinant human CD14, a neutralizing antibody to human CD14 (1 μg/mL, clone 134620) or isotype IgG_1 (clone 11711) for 30 minutes, followed by stimulation with LPS (10 ng/mL for IPE, 10 μg/mL for ARPE-19—concentrations known to induce maximum cytokine secretion). Culture supernatants were collected at 24 hours, and CXCL8 (IL-8) concentrations were determined with a commercially available ELISA (Human CXCL8/IL-8 DuoSet ELISA; R&D Systems). CXCL8 was assayed because it is a typical epithelialum-derived proinflammatory mediator that is secreted by LPS-treated IPE and ARPE-19 cells.

TLR2 and TLR4 Neutralization Study

IPE and ARPE-19 were seeded into 24-well plates and subjected to serum starvation as just described. The cells were placed in SFM with 10 μg/mL of neutralizing antibodies to TLR2 (clone TL2.1), TLR4 (clone HTA125), or isotype control (IgG_{2a}) for 30 minutes, followed by stimulation with LPS for 24 hours. CXCL8 concentration in the culture supernatant was determined by ELISA.

Statistical Analysis

Experiments were performed in triplicate, and the results expressed as the mean ± SD. One-way ANOVA was used to compare responses between different treatment groups. A two-tailed $P < 0.05$ was considered significant (Prism, ver. 5; GraphPad Software, San Diego, CA).

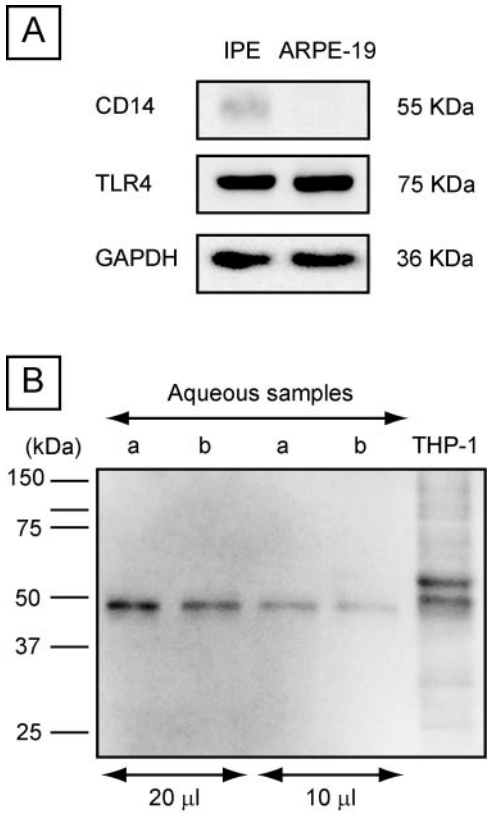

FIGURE 3. Western blot analysis of ocular pigment epithelial cells and normal aqueous. Whole-cell lysates (20 μg) or normal human aqueous (10–20 μL) were separated by 10% SDS-PAGE, transferred onto PVDF, and probed with antibodies to CD14, TLR4, and GAPDH (**A**) or anti-CD14 alone (**B**). IPE and ARPE-19 cells expressed equal amounts of TLR4 and GAPDH, but CD14 was detected only in IPE cell lysates (**A**). sCD14 was detected in aqueous samples (a and b) as a single 50-kDa band, whereas mCD14 from the positive control THP-1 cell lysate migrated at 53 and 55 kDa (**B**). Blots are representative of two independent experiments.

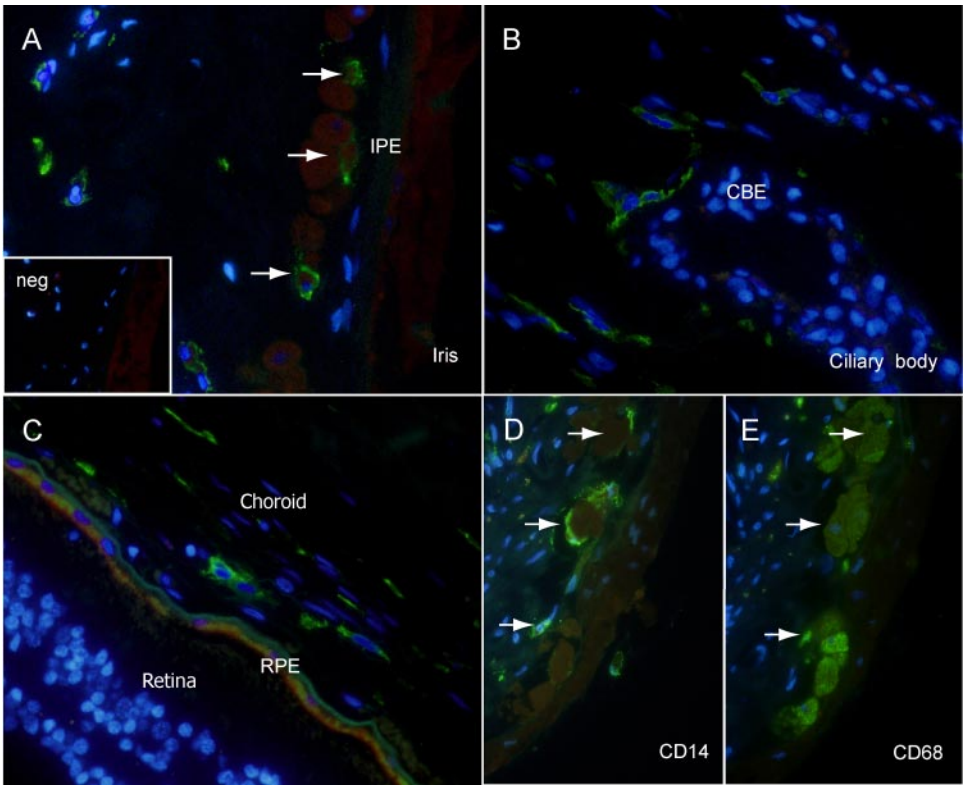

FIGURE 4. CD14-immunoreactive cells in normal human uveal tissues. Four-micrometer paraffin-embedded sections were incubated with anti-CD14 (**A**–**D**), anti-CD68 (**E**) or isotype control (neg, *inset*), followed by Alexa Fluor 488–conjugated goat anti-mouse IgG. Specific labeling is denoted by *green* fluorescence and nuclei counterstained in *blue*. CD14+ cells are present within the iris, ciliary body, and choroid. Some pigmented cells (*arrows*) stained for CD14 (**A**, **D**) and for CD68 in an adjacent section (**E**). Original magnification, ×600.

RESULTS

Expression of LPS Receptor Complex by Human IPE

In vitro expression of TLR2, TLR4, MD-2, and CD14 by IPE and ARPE-19 was examined by RT-PCR (Fig. 1) and by different protein assays (Figs. 2, 3). Technical problems arising from our trypsin digestion protocol caused CD14 expression in the cultured cells to be undetectable by flow cytometry (data not shown). However, CD14 was detected in IPE and ARPE-19 by RT-PCR and immunohistochemistry (Figs. 1, 2) and by Western blot for IPE (Fig. 3A), with IPE expressing more CD14 than ARPE-19 cells. These results imply that both cell types possess the necessary components to sense LPS.

CD14 Expression in Normal Human Uveal Tissues and Aqueous

In normal uveal tissues, strong CD14 immunoreactivity was noted in immune cells located within the stroma of the iris, ciliary body, and choroid (Figs. 4A–C). A small number of pigmented cells in the posterior iris showed mCD14 staining (Fig. 4A, arrows). CD14+ pigmented cells in the iris were CD68+ in adjacent sections (Figs. 4C, 4D, respectively) suggesting that they are macrophages. CD207 staining was absent in all uveal tissues but was observed in positive control skin tissue (data not shown), therefore CD14+ cells in the normal uvea are unlikely to be mature langerin-expressing dendritic cells. IPE and CBE did not exhibit CD14-immunoreacitivity in vivo, but we cannot comment on the CD14 staining in the RPE, because of the strong autofluorescence exhibited by these cells. In Western blot analysis, we detected sCD14 in normal aqueous as a single 50-kDa band under nonreducing conditions, whereas mCD14 from THP-1 cells migrated as a doublet at 53 and 55 kDa (Fig. 3B).

LPS-Induced IPE Secretion of Proinflammatory Cytokines and Chemokines

IPE and ARPE-19 cells secreted a panel of cytokines, chemokines, and growth factors at baseline and when stimulated with LPS (Fig. 5, Table 3). Unstimulated IPE secreted IL-6, CXCL8, CXCL10, CCL2, CCL3, CCL4, CCL5, PDGF, and VEGF, whereas ARPE-19 secreted only CXCL8, CCL2, and VEGF. When exposed to LPS, IPE secreted enhanced levels of IL-6, CXCL8, CXCL10, CCL2, CCL4, and CCL5, whereas LPS-treated ARPE-19 responded with elevated secretion of IL-6, CXCL8, and CCL2. With the exception of VEGF (Figs. 5D, 5H), IPE responded to lower levels of LPS when compared with ARPE-19, which only secreted significant amounts of cytokines when stimulated with high doses of LPS. Furthermore, IPE secreted an additional repertoire of chemokines that were undetected in ARPE-19 culture supernatant (Figs. 5I–L). IL-1β, IL-10, IL-12, IL-17, TNF-α, and IFN-γ were not detected in supernatants of either cell type.

CD14-Induced Modulation of the LPS Response of Human IPE

The effect of CD14 on the LPS response of IPE and ARPE-19 cells was investigated with the addition of recombinant CD14 (Figs. 6A, 6B). ARPE-19 cells, which expressed low levels of endogenous CD14, secreted significantly elevated levels of CXCL8 in the presence of 1 μg/mL of recombinant CD14 (levels typically found in serum).[33] In contrast, IPE cells expressing high levels of endogenous CD14 did not respond to additional CD14, irrespective of the concentrations tested.

The contribution of endogenous CD14 to the LPS response in both cell types under serum-free conditions was investigated by using a neutralization antibody to human CD14. Pretreatment with a CD14-neutralizing antibody attenuated LPS-in-

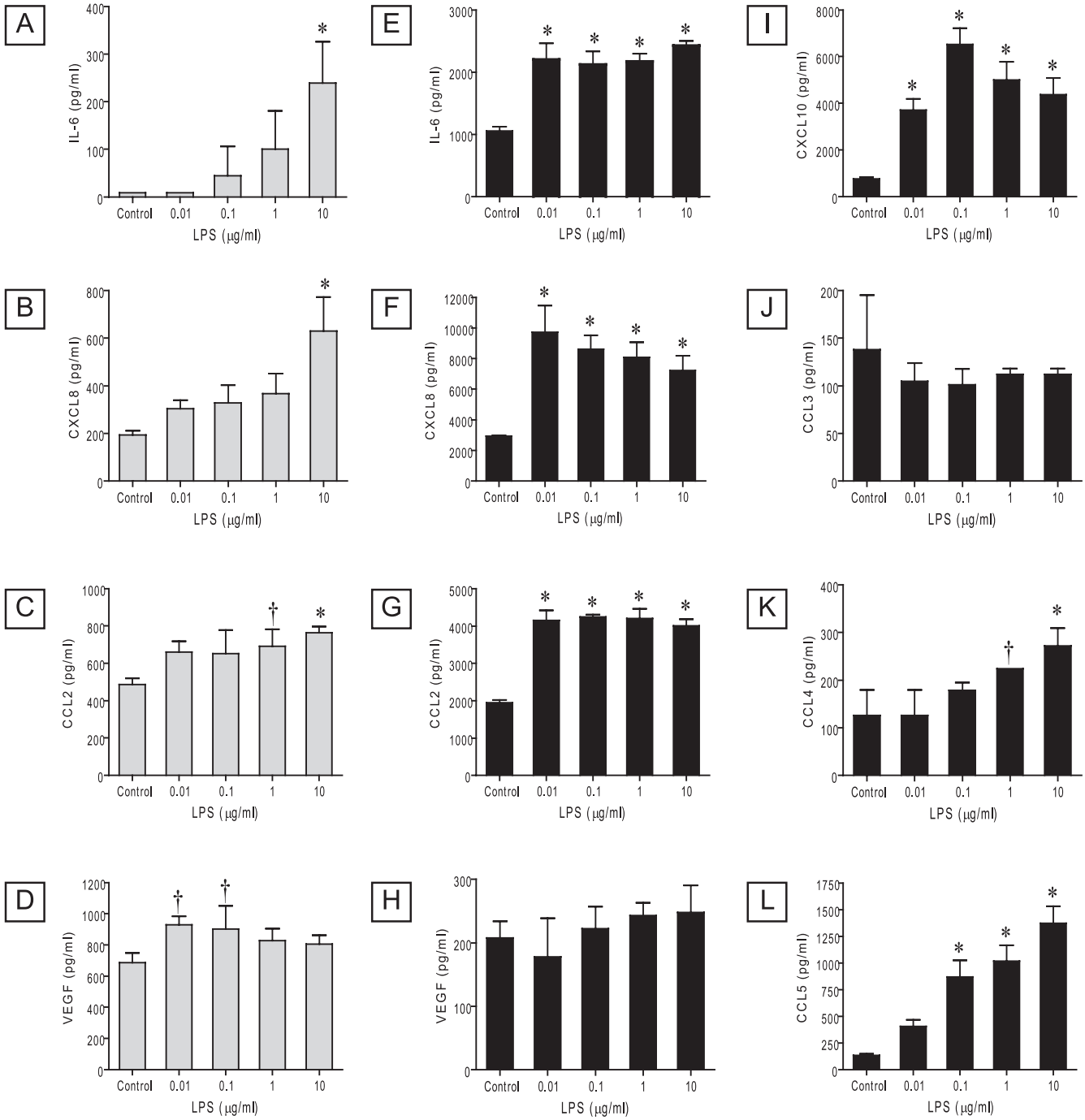

FIGURE 5. Proinflammatory mediators secreted by ocular pigment epithelial cells. ARPE-19 (▨, **A–D**) and IPE (■, **E–L**) cells were exposed to 0.01 to 10 μg/mL of LPS or SFM (control). At 24 hours, conditioned media were collected and analyzed for cytokines and chemokines, by multiplex bead array assay. Data are expressed as the mean ± SD ($n = 3$). One-way ANOVA and Dunnett's post test were used to compare LPS-treated cells to controls; $^*P < 0.01$, $\dagger P < 0.05$.

duced CXCL8 secretion from IPE cells (Fig. 6C), but this was not apparent in ARPE-19 cells (Fig. 6D).

Inhibition of the LPS Response in IPE

In TLR2 and -4 antibody neutralization studies, secretion of CXCL8 from IPE in response to LPS treatment was inhibited in the presence of anti-TLR4 but not by anti-TLR2 or isotype control (Fig. 7A). In contrast, CXCL8 secretion by LPS-treated ARPE-19 was unaffected by neutralizing antibodies or their isotype control IgG (Fig. 7B).

DISCUSSION

We showed, for the first time, that IPE expresses a functional LPS receptor complex and, independent of other cell types, secretes proinflammatory mediators when exposed to LPS. The cytokines released by IPE (IL-6, CXCL8, CXCL10, CCL2, CCL4, and CCL5) are similar to those present in the aqueous of patients with AAU[1,43] suggesting that IPE is a source of cytokines during active disease. However, a lack of measurable IL-1β and TNF-α from our culture supernatants is unexpected,

TABLE 3. Cytokines, Chemokines, and Growth Factors Secreted by LPS-Stimulated IPE and ARPE-19

Cytokine/Chemokine	IPE	ARPE-19
IL-6	↑	↑
CXCL8 (IL-8)	↑	↑
CXCL10 (IP-10)	↑	ND
CCL2 (MCP-1)	↑	↑
CCL3 (MIP-1 α)	→	ND
CCL4 (MIP-1 β)	↑	ND
CCL5 (RANTES)	↑	ND
VEGF	→	↑
PDGF	→	ND

↑, induced; →, no change; ND, not detected.

given that these cytokines are known to be released on NF-κB activation downstream of LPS stimulation. Certainly, in animal models of EIU, others have detected IL-1β and TNF-α from ocular tissues and serum.[44,45] One explanation may be that unlike immune cells, LPS-stimulated ocular pigment epithelium do not produce IL-1β or TNF-α. Supporting this view, Leung et al.[46] also failed to detect IL-1β or TNF-α in culture supernatants of LPS-stimulated primary RPE and ARPE-19. Others have reported that IPE and RPE do not express TNF-α transcript[39] and preferentially produce IL-1α rather than IL-1β.[47] Our observations may also reflect a limitation of the in vitro model, since gene expression of cultured RPE cells differs from that of native (laser-captured) RPE.[48] Finally, insufficient sensitivity of IL-1β and TNF-α assays may be another explanation, since detection of these cytokines has been inconsistent in clinical studies on uveitis. One group reported IL-1β and TNF-α to be below detection limits in the aqueous of patients with active uveitis,[43] whereas another detected both cytokines in the aqueous of patients with idiopathic uveitis, where concentrations did not differ significantly from noninflamed cataract control aqueous.[49] In children with uveitis, elevated aqueous TNF-α was reported, but IL-1 concentrations did not differ between uveitis and control groups.[50] More clinically relevant cytokines would be CXCL8 and CCL2, given their early appearance in the aqueous and correlation with disease severity,[51,52] whereas no correlation was observed for TNF-α.[52] Therefore, although IPE may respond to LPS with a limited set of cytokines, those produced are clinically important in dictating disease severity.

IPE expressed more CD14 than ARPE-19, which may explain their increased responsiveness to LPS. In IPE cells, the LPS response was CD14- and TLR4-dependent, whereas ARPE-19 cells secreted cytokines even in the presence of CD14- or TLR4-neutralizing antibodies. It is possible that cell immortalization accounts for these differences. In studies comparing ARPE-19 to primary RPE cells, elevated expression of proteins associated with microtubule cytoskeleton and IL-18 production was noted in ARPE-19 cells.[53,54] Therefore, a better control for primary IPE cells would be donor-matched primary RPE. Despite our limitations, some conclusions may still be drawn, since others have reported that primary RPE and ARPE-19 cells respond in a manner similar to LPS by secreting the same set of cytokines (IL-6, IL-8, and MCP-1).[46]

Under our experimental conditions, sCD14 amplification of cytokine secretion in LPS-treated ARPE-19 cells occurred at concentrations similar to those in normal serum (1.5–1.9 μg/mL),[27] rather than that reported in tears (561.1 ± 281.6 ng/mL).[26] However, cytokine secretion was also higher in SFM without additional sCD14, when compared with cells exposed to low levels of sCD14 (Fig. 6B). Although sCD14 is known to augment TLR4 signaling, it may also function as a decoy for LPS. For instance, excess sCD14 is shown to reduce monocyte

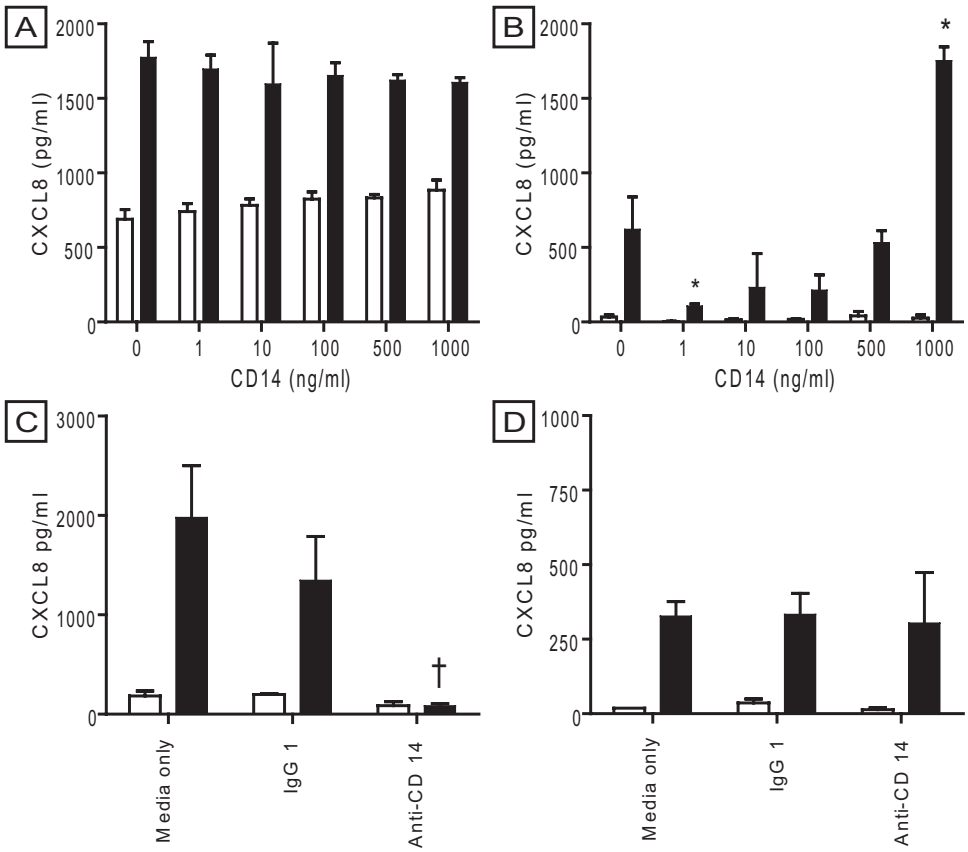

FIGURE 6. CD14-modulated LPS-induced CXCL8 secretion in ocular pigment epithelial cells. IPE (**A**, **C**) and ARPE-19 (**B**, **D**) cells were stimulated with LPS (■) or SFM (□), in the presence of additional recombinant CD14 (**A**, **B**) or a CD14 neutralization antibody (**C**, **D**). At 24 hours, CXCL8 secretion was measured by ELISA. Data are expressed as the mean ± SD ($n = 3$); one-way ANOVA and Bonferroni's posttest were used to analyze the results. (**B**) LPS-treated ARPE-19 cells secreted significantly higher amounts of CXCL8 in the presence of 1000 ng/mL of sCD14 when compared with LPS-treated cells in the SFM control (*$P < 0.01$). (**C**) The LPS response of IPE cells was significantly suppressed in the presence of anti-CD14 IgG when compared with cells stimulated in medium alone (†$P < 0.001$).

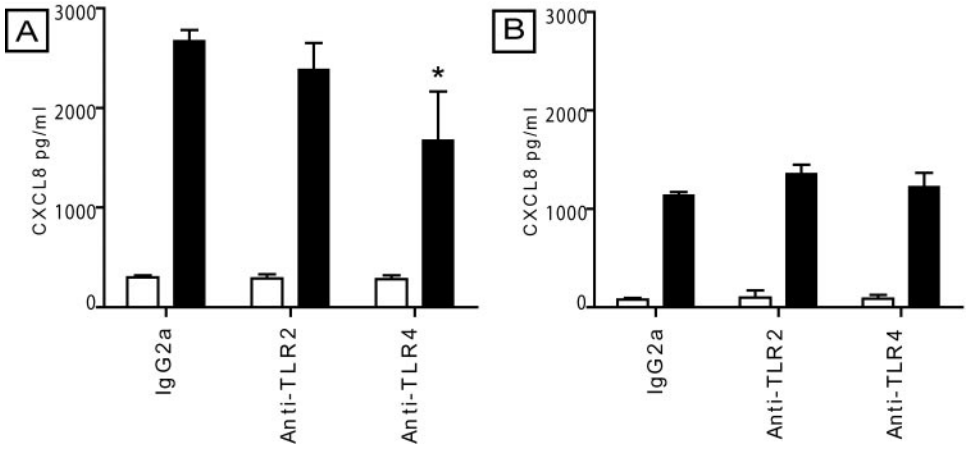

FIGURE 7. Inhibition of LPS-induced CXCL8 secretion after TLR4 neutralization. IPE (**A**) and ARPE-19 (**B**) cells were stimulated with LPS (■) or exposed to SFM (□) in the presence of neutralizing antibodies to TLR2, TLR4, or the isotype control (IgG2a) at 10 μg/mL. Supernatants were assayed for CXCL8. Data represent the mean ± SD ($n = 3$), One-Way ANOVA and Bonferroni's posttest were used to analyze the data. CXCL8 secretion by LPS-treated IPE was significantly suppressed in the presence of a TLR4-neutralizing antibody when compared with cells exposed to an appropriate isotype antibody ($^*P < 0.01$), whereas cytokine secretion was not suppressed by anti-TLR2 antibody.

activation by LPS[55] and to inhibit LPS-induced TNF-α production in whole blood.[56] Furthermore, transgenic mice expressing high levels of sCD14 paradoxically showed resistance to LPS.[57] Indeed, transfer of LPS from MD-2 to sCD14 can occur and may account for the attenuation of the LPS response.[58] This mechanism may explain why the LPS response is partly suppressed in ARPE-19 cultures exposed to lower concentrations of sCD14 in comparison to cells in SFM. We speculate that low concentrations of sCD14 serve a physiological role to protect ocular structures from inappropriate activation of the inflammatory cascade.

Of interest, LPS-treated ARPE-19 cells secreted CXCL8 in the absence of sCD14 (Fig. 6D). It is tempting to hypothesize that a CD14-independent mechanism is present to compensate for low CD14 expression in these cells. However, it should be noted that minor contaminants in the commercially purchased LPS stock could also explain our observations. In support of the CD14-independent signaling concept, others have reported LPS uptake in CD14-knockout endothelial cells.[59] The concentration and type of LPS may also alter the mechanism of TLR4 activation. For instance, CD14-independent signaling is more prominent when LPS concentration is high,[60] and in wild-type LPS lacking a typical O-antigen, with short carbohydrate chains.[61] Further studies examining the response of uveal cells to LPS from different organisms may be useful, given that uveitis is associated with specific pathogens such as *Klebsiella*, *Salmonella*, *Shigella*, *Yersinia*, and *Chlamydia trachomatis*.[3]

In this study, we have shown by three independent methods that IPE cultures expressed CD14. Conversely in uveal tissues, CD14 immunoreactivity was localized to CD68+ macrophages but not IPE cells. Nor did we observe the CD14 staining in CBE cells that has been reported by other researchers.[23] Different sensitivities between the detection methods or cell culture upregulation of CD14 in IPE cells may explain these discrepancies. To address the latter would require comparison of CD14 expression by freshly isolated IPE and subsequent derivative cultures. Although IPE may lack mCD14 in vivo, we have demonstrated the presence of sCD14 in normal aqueous. In addition, others have detected CD14 mRNA by RT-PCR in the aqueous of patients with uveitis and age-related cataract,[62] and CD14+ cells were reported in the aqueous of patients with active uveitis.[63,64] Collectively, these observations suggest that CD14 is present in normal uveal tissue and may augment the LPS response in cells that express little to no endogenous CD14. In future studies, it would be of interest to quantify the levels of intraocular sCD14 in normal and inflamed eyes, given its role in dictating ocular pigment cell response to LPS.

In conclusion, our discovery that IPE cells in the human uvea are equipped with innate pattern-recognition receptors such as TLR4 and CD14 that respond to LPS extends the current understanding of the role of microbial triggers and uveal innate immune mechanisms in the pathogenesis of AAU. Furthermore, components of the LPS receptor complex could be useful targets for therapeutic strategies for this disease.

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