TLR4 Mediates Human Retinal Pigment Epithelial Endotoxin Binding and Cytokine Expression

Susan G. Elner,[1] *Howard R. Petty,*[1] *Victor M. Elner,*[1,2] *Ayako Yoshida,*[1] *Zong-Mei Bian,*[1] *Dongli Yang,*[1] *and Andrei L. Kindzelskii*[1]

Purpose. It was previously demonstrated that toll-like receptor 4 (TLR4) is involved in species-specific human retinal pigment epithelial (HRPE) photoreceptor outer segment recognition and oxidant production. This study was performed to demonstrate the classical role of TLR4 in HRPE endotoxin (lipopolysaccharide; LPS) binding leading to HRPE proinflammatory cytokine secretion.

Methods. Cultured HRPE cells were examined for TLR4 expression by immunofluorescence, Western blot analysis, and RT-PCR. HRPE cells labeled with fluorescent monoclonal antibodies (mAbs) to TLR4 and its associated adhesion molecule, CD14, were analyzed by real-time microscopy and resonance energy transfer (RET), determining associations of fluorescent LPS, TLR4, and CD14. LPS-induced HRPE secretion of interleukin (IL)-8 was measured with and without specific blocking mAb to TLR4 and CD14. HRPE TLR4 expression was measured after LPS exposure in the presence and absence of blocking antibodies to TLR4 and CD14.

Results. All three HRPE cell lines demonstrated constitutive TLR4 expression by immunofluorescence, Western blot analysis, and RT-PCR. Examination of HRPE cells labeled with fluorescent mAb to TLR4, CD14, and LPS demonstrated RET among the three molecules, indicating that LPS-CD14 binding preceded LPS-TLR4 binding and the close association of CD14 and TLR4. LPS-induced IL-8 was significantly reduced ($P < 0.05$) in the presence of blocking mAb to TLR4 or CD14. Upregulation of HRPE TLR4 gene and protein expression occurred in response to LPS exposure and was inhibited by mAb to TLR4 or CD14.

Conclusions. HRPE TLR4 is a multifunctional molecule that participates in photoreceptor outer segment membrane recognition, oxidant production, LPS recognition, and cytokine production. These roles indicate potential involvement in retinal degenerative and inflammatory processes. (*Invest Ophthalmol Vis Sci.* 2005;46:4627–4633) DOI:10.1167/iovs.05-0658

The retinal pigment epithelium (RPE), which forms the outer blood-retina barrier, acts as an intermediary between the systemic circulation and the neurosensory retina, which relies on RPE functions for its homeostasis. The RPE expresses surface receptors mediating innate and antigen-specific immunity that may be important in ocular defense and may participate in retinal disease mechanisms. These receptors bind specific ligands, leading to the selective, rapid, and efficient engulfment of particulates or intercellular binding and RPE activation. RPE innate immune receptors include CD11b/CD18 (CR3; iC3b) and CD11c/CD18 (CR4) complement receptors,[1] lipopolysaccharide (LPS) receptor CD14,[2] pattern recognition receptor toll-like receptor 4 (TLR4),[3] and scavenger receptors type I/II,[4,5] CD68,[6] and CD36.[7,8] RPE receptors mediating antigen-specific immune responses include CD16 (FcγRIII) immunoglobulin,[1] HLA-DR,[9] and intercellular adhesion molecule-1 (ICAM-1).[6] Ambient proinflammatory cytokines, including IFN-γ, TNF-α, and IL-1β, upregulate the expression of RPE HLA-DR[9] and ICAM-1[6] and the secretion of numerous RPE cytokines, including IL-8, an important RPE C-X-C chemokine that attracts and activates neutrophils and eosinophils.[10,11] The RPE expression of these receptors and potent proinflammatory cytokines, including IL-8, implies important roles for RPE cells in ocular defense.

CD14, the primary receptor for LPS, is a 55-kDa glycosyl phosphatidylinositol (GPI)-anchored glycoprotein initially identified on the surfaces of mononuclear phagocytes, neutrophils, and RPE cells.[2,12–14] Inasmuch as CD14 lacks an intracellular domain to transmit an activation signal into the cell, transmembrane CD14 signaling requires accessory membrane-linked coreceptors, the best characterized of which is TLR4.[15,16] LPS binding induces transmembrane signals,[17,18] leading to nuclear factor (NF)-κB[19,20] activation that results in cytokine production, including IL-8.[2,21–23]

In addition to LPS, CD14 binds a diverse array of other bacterial, viral, fungal, and host components, consistent with its role in innate immunity.[18,24–26] This has led to the recognition of CD14 as a pattern recognition receptor imparting innate immunity to a broad spectrum of infectious agents.[16,18,24] Besides its role in host defense, CD14 may subserve other host-related biologic functions. For example, CD14 appears to be involved in the regulation of apoptosis and apoptotic cell clearance,[27–29] in the exchange of different phospholipids, and in monocyte-endothelial interactions.[16,30]

TLR4, which is known to transduce CD14 responses to LPS, is also essential for innate immunity against invading pathogens.[31–33] Like CD14, TLR4 is one of the pattern recognition receptors, which bind exogenous and endogenous substances. Specifically, human TLR4 participates in cellular responses to exogenous substances, including LPS of Gram-negative bacteria, lipoteichoic acid of Gram-positive bacteria, and the F protein of respiratory syncytial virus, and it is a receptor for the endogenous substances HSP60 (and certain homologous proteins), the fibronectin extra domain A, and hyaluronan.[34–36]

We previously reported human RPE (HRPE) immunohistochemical, genetic, and functional expression of CD14 in response to LPS[2] and the novel role of HRPE TLR4 in transmembrane signaling in response to photoreceptor outer segment binding.[37] In this study, we investigated the role of HRPE TLR4 in LPS binding and cytokine signaling and the effect of LPS on HRPE TLR4 expression. TLR4 HRPE expression, in

From the Departments of [1]Ophthalmology and [2]Pathology, University of Michigan, W. K. Kellogg Eye Center, Ann Arbor, Michigan.

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Corresponding author: Andrei L. Kindzelskii, Department of Ophthalmology, University of Michigan, W. K. Kellogg Eye Center, 1000 Wall Street, Ann Arbor, MI 48105; andreik@umich.edu.

conjunction with CD14 expression at the blood-retina barrier, may play roles in ocular defense and other pathophysiologic mechanisms.

Materials and Methods

HRPE Cell Culture

HRPE cells were isolated from eyes of healthy donors within 24 hours of death, as previously described, in accordance with the Declaration of Helsinki.[38] In brief, the sensory retina was separated gently from the HRPE monolayer, and the HRPE cells were removed from Bruch's membrane using a 1-hour incubation with papain (5 μg/mL; Sigma Chemical Co., St. Louis, MO). Isolated HRPE cells were seeded into flasks (Falcon Primaria; Becton-Dickinson Inc., Lincoln Park, NJ) in Dulbecco's modified essential medium (DMEM; Sigma) containing 15% fetal bovine serum (Sigma), penicillin G (100 U/mL; Sigma), streptomycin sulfate (100 μg/mL; Sigma), and amphotericin B (0.25 μg/mL; Sigma). Cultured HRPE cells formed monolayers showing typical polygonal morphology, pigmentation of scattered cells, and uniform immunohistochemical staining for cytokeratin characteristic of these epithelial cells.[39] In all experiments, simultaneous, parallel assays were performed on second- to fourth-passage cells seeded at the same time and density from the same parent cultures. All experiments were repeated at least three times on different HRPE cell lines. HRPE cultures were maintained in the media until used for TLR4 and CD14 immunofluorescence staining, TLR4 semiquantitative PCR, TLR4 Western blot analysis, or cytokine ELISA.

HRPE Cell Stimulation with Endotoxin

Before experiments, HRPE cells were incubated in fresh LPS-free medium for 24 hours. HRPE cells were then incubated in LPS-free medium or in the same medium containing 100 ng/mL LPS (*Escherichia coli* serotype 0111:B4; Sigma). In some experiments, polymyxin (10 μg/mL; Sigma) was used to antagonize the effects of LPS. After experimental incubations, conditioned medium (CM) were collected and centrifuged to remove particulates. CM was stored at −70°C until ELISA was performed.

Aliquots of all media and reagents were tested using a limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD) whose LPS sensitivity was <1 pg/mL to exclude the possibility of LPS contamination and to ensure that neither cytokine production nor TLR4 stimulation was caused by the presence of contaminating LPS. Blocking anti-CD14 (clone UCHM-1; A-306; Sigma) or anti-TLR4 (clone HTA125; MCA2061XZ; Serotec, Raleigh, NC) mAb was included in selected assays to antagonize the effects of LPS.

Fluorescence Demonstration of LPS, TLR-4, and CD14 Association on HRPE Cells

Fluorescein isothiocyanate (FITC) and tetramethylrhodamine (TRITC) were obtained from Molecular Probes (Eugene, OR). EDTA was obtained from Fisher Scientific Co. (Fairlawn, NJ). Conjugation of LPS with FITC and analysis of the FITC-LPS conjugate ability to activate leukocyte NAD(P)H oscillations were performed as previously described.[40]

For fluorescence studies, mouse anti–human CD14 (clone CRIS-6) and anti–human TLR4 (clone HTA1216) mAb were obtained from BioSource International (Camarillo, CA) and Kensuke Miyake (Saga Medical School, Saga, Japan), respectively. $F(ab')_2$ fragments of anti-CD14 and anti-TLR4 mouse mAb were prepared as previously described.[41–47] FITC-conjugated CD14 and TRITC-conjugated TLR4 mAb were prepared as previously described.[48,49] Fluorescence conjugates were separated from unreacted fluorochromes by column chromatography (Sephadex G-25; Sigma). Purified conjugates were dialyzed against PBS at pH 7.4 overnight at 4°C.

HRPE cells, grown to confluence on coverslips, were labeled with nonsaturating concentrations of $F(ab')_2$ fragments of FITC-conjugated anti-CD14 or TRITC-conjugated anti-TLR4 mAb (both 100 ng/mL) for 30 minutes at 37°C in phenol red-free culture medium. After washing four times with phenol red-free Hanks' balanced salt solution (HBSS), cells were incubated in phenol red-free medium for 30 minutes at 37°C. In some preparations, the cells were washed twice with cold phenol red-free HBSS and then labeled with FITC-LPS (100 ng/mL) for 20 minutes at 4°C. Cells were then incubated for 30 minutes at 37°C. After staining, the cells were washed twice with cold HBSS. Coverslips were then transferred to a microscope stage held at 37°C. These experimental manipulations had no apparent effect on cell activation or shape change as assessed by differential image contrast (DIC) microscopy and right-angle light scatter, as previously described.[50]

An inverted fluorescence microscope with HBO-100 mercury illumination (Axiovert; Carl Zeiss, New York, NY) interfaced to a computer workstation (Dell 410; Dell Inc., Round Rock, TX) through a video card (Scion SG-7; Vay Tek, Fairfield, IA) was used for cell examination. Fluorescence images were collected by an intensified charge-coupled device camera (model XC-77; Hamamatsu, Hamamatsu City, Japan) and processed with image software (Scion Image Software; Vay Tek). A narrow bandpass-discriminating filter set was used with excitation at 485DF20 nm and emission of 530DF30 nm for FITC. For TRITC, an excitation of 540DF20 nm and an emission of 590DF30 nm were used (Omega Optical, Brattleboro, VT). Long-pass dichroic mirrors at 510 and 560 nm were used for FITC and TRITC, respectively. For resonance energy transfer (RET) microscopy,[51] the 485DF22 narrow bandpass discriminating filter was used for excitation, and the 590DF30 filter was used for emission with a 510-nm long-pass dichroic mirror.[45,49,51] DIC images were collected using polarizers (Carl Zeiss) and a charge-coupled device camera (Dage-MTI, Michigan City, IN). Background-subtracted digitized images were averaged and then electronically stored.

Quantitative microfluorometry was used to evaluate RET levels. This was performed using a cooled high-sensitivity photomultiplier tube in a detection system (D104; Photon Technology International, Inc.) attached to a microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).[52–54]

Semiquantitative Reverse-Transcriptase–Polymerase Chain Reaction for TLR4

Synthetic oligonucleotide primers based on the cDNA sequences of human *TLR4* and β-actin were prepared (*TLR4*, 5′-TCCCTC-CAGGTTCTTGATTACAGTC-3′ and 5′-TGCTCAGAAACTGC-CAGGTCTG-3′; *β-actin*, 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′). Total RNA was extracted by using reagent (TRIzol; Gibco BRL, Carlsbad, CA), according to the manufacturer's procedure. RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The cDNA was denatured for 5 minutes at 94°C, followed by 28 PCR cycles. Each cycle included 1-minute denaturation at 94°C, 1-minute primer annealing at 55°C, and 2-minute polymerization at 73°C. Each RT-PCR reaction mixture was analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Western Blot Analysis for TLR4

HRPE cells were lysed with buffer containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 0.15 M sodium chloride, 10% glycerol, 1.5 mM magnesium chloride, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM AEBSF, 10 mM sodium fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Lysates were then incubated on ice for 15 minutes with shaking. Then the extracts were centrifuged at 18361*g* for 15 minutes at 4°C.

Western blot analysis of cellular extracts from HRPE cells was performed according to the manufacturer's procedure. Briefly, samples containing 20 μg protein were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then were electrotransferred to nitrocellulose membranes. For signal protein detection, samples were blocked with a solution of Tris-buffered saline containing

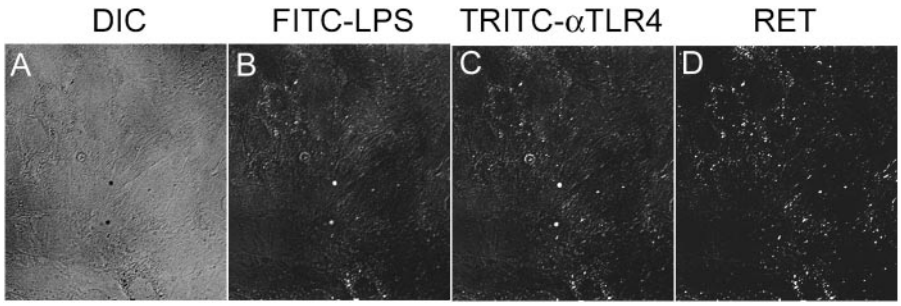

FIGURE 1. TLR4 and LPS co-localization on live HRPE cells. (**A**) DIC micrograph of cultured HRPE cells in confluent cell cultures. (**B**) Cell surface binding of FITC-labeled LPS. (**C**) Cell surface fluorescence resulting from bound TRITC-labeled anti-TLR4 (αTLR4) in the presence of bound FITC-labeled LPS. The pattern of TLR4 labeling is similar in distribution to that observed for LPS binding. (**D**) RET between fluorochromes labeling TLR4 and LPS indicates close approximation of the molecules to within 7 nm. No significant RET was present in the absence of LPS.

5% dry milk and 0.1% Tween-20 (TBST) at room temperature for 1 hour, probed with anti-TLR4 mAb (HTA125; Serotec), and washed three times in TBST. Membranes were incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature and washed three times with TBST. Membranes were then visualized using an enhanced chemiluminescence technique.

Enzyme-Linked Immunoabsorbent Assay for Interleukin-8

Antigenic IL-8 was quantitated using a double-ligand ELISA method, as described previously.[55] Briefly, ELISA was performed on serial dilutions of HRPE CM. CM was aspirated from the surfaces of the HRPE monolayers and centrifuged to remove cell particulates. Cell lysates were obtained by lysing HRPE monolayers with distilled water.[56] Mouse anti–human IL-8 mAb (clone 6217.111; MAB208) and biotinylated polyclonal goat anti–human IL-8 (clone BAF208) were used as capture and detection antibodies, as directed by the manufacturer (R&D Systems, Minneapolis, MN). This ELISA method consistently detected IL-8 concentrations of >10 pg/mL. Standards included 0.5 log dilutions of recombinant human IL-8 (R&D Systems) from 5 pg/well to 100 ng/well.

Statistical Analysis

Individual experiments were performed on three different HRPE cell lines. Each cell line displayed similar fold-increases or decreases over control levels. Data are expressed as mean ± SD. Various assay conditions were evaluated using analysis of variance test with a post hoc analysis (Schiff multiple comparison test); $P < 0.05$ was considered statistically significant.

RESULTS

LPS, TLR4, and CD14 Co-localization on Live HRPE Cells

To demonstrate TLR4 and LPS co-localization, imaging was performed on live, confluent HRPE cultures as examined by DIC microscopy (Figs. 1A, 2A, 2E). HRPE cells labeled with TRITC–anti-TLR4 mAb, but not labeled idiotypic mAb, demonstrated delicate staining that was enhanced along intracellular interfaces (Figs. 1C, 2C, 2G), confirming our previous observations of HRPE TLR4 expression.[37] Simultaneous FITC-LPS labeling of HRPE cultures demonstrated discrete cell surface binding (Fig. 1B) that was similar in distribution to that obtained with TRITC–anti-TLR4. Strong RET between the two fluorochromes on HRPE cells labeled with FITC-LPS and TRITC–anti-TLR4 mAb (Fig. 1D) yielded photochemical confirmation of the proximity of the two molecules to within 7 nm of each other on the HRPE surface.

Simultaneous labeling of HRPE cultures with FITC–anti-CD14 and TRITC–anti-TLR4 mAb was then performed in the presence and absence of LPS (Fig. 2). Under both conditions, discrete immunolabeling of live HRPE cells revealed similar discrete localization of CD14 (Figs. 2B, 2F) and TLR4 (Figs. 2C, 2G), suggesting that endotoxins were necessary for CD14-TLR4 receptor aggregation on HRPE cells. No RET was detected in HRPE cultures labeled with FITC-LPS, FITC–anti-CD14 mAb, or TRITC–anti-TLR4 mAb alone or with simultaneous labeling with irrelevant control FITC- or TRITC–anti-idiotypic mAb (data not shown).

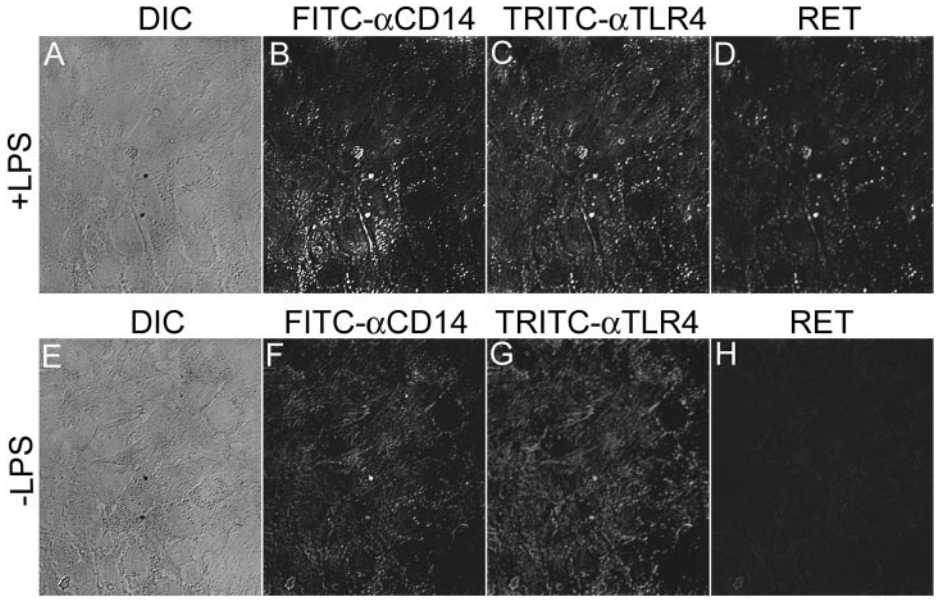

FIGURE 2. TLR4 and CD14 co-localization on live HRPE cells. (**A**, **E**) DIC micrographs of cultured HRPE cells in confluent cell cultures. (**B**, **F**) Cell surface binding of FITC-labeled anti-CD14 (αCD14). (**C**, **G**) Cell surface fluorescence resulting from bound TRITC-labeled anti-TLR4 (αTLR4) in the presence of bound FITC-labeled anti-CD14 with (**B**) and without (**F**) the presence of LPS. The pattern of TLR4 labeling is similar in distribution to that observed for CD14 binding. (**D**, **H**) RET between fluorochromes labeling TLR4 and CD14 indicates close approximation of the molecules to within 7 nm in the presence of LPS (**D**), but not in the absence of LPS (**H**).

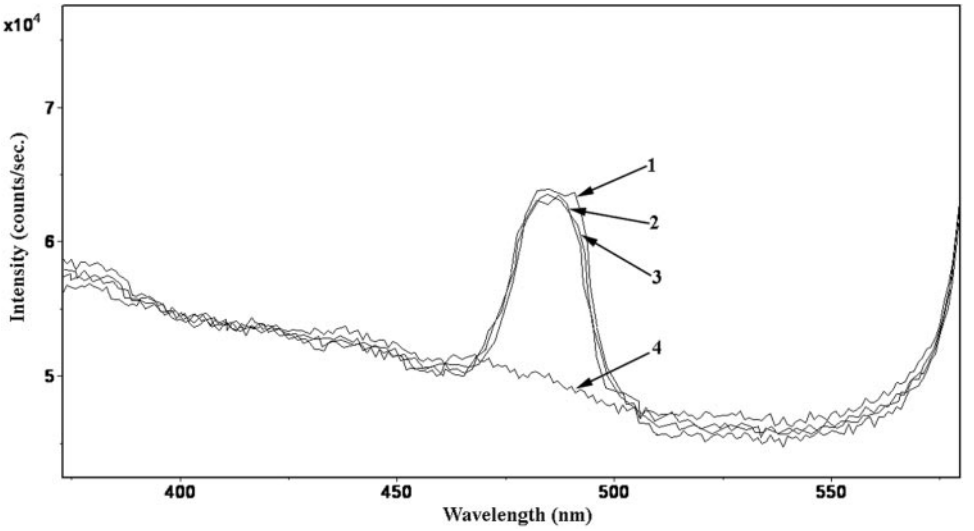

FIGURE 3. RET among TLR4, CD14, and LPS. In the presence of LPS, excitation at 488 nm results in peak RET fluorescence measured at 590 nm when HRPE cells are co-labeled, as indicated in Figures 1 and 2, with fluorescent mAb to CD14 and TLR4 (*arrow 1*), CD14 and LPS (*arrow 2*), and TLR4 and LPS (*arrow 3*). RET indicates simultaneous, close approximation of all three molecule pairs at steady state because of 488 nm light-induced FITC 520 nm emission, which is capable of exciting TRITC to emit 590 nm light only if the molecule pairs are in close approximation. No RET between CD14 and TLR4 is found in the absence of LPS (*arrow 4*).

Steady State Excitation and Dynamic Emission RET for HRPE LPS, TLR4, and CD14 Co-localization

Excitation RET was performed to show close approximation of LPS, TLR4, and CD14 pairs (Fig. 3). Peak excitation of FITC at 488 nm (measured in abscissa in Fig. 3) resulted in peak fluorescence at 590 nm (measured in ordinate in Fig. 3) by the HRPE cultures, also labeled with TRITC, only when the molecule pairs labeled with FITC and TRITC were extremely close, within 7 nm of each other, thereby permitting 488 nm induced FITC emission at 520 nm to excite TRITC and to result in TRITC emission at 590 nm. As seen in Figure 3, simultaneous labeling of CD14/LPS and TLR4/LPS with FITC and TRITC (curves 2, 3) resulted in weak, steady state RET at 590 nm when the HRPE cultures were illuminated with 488 nm light. However, steady state CD14/TLR4 RET was only observed in the presence of LPS (curve 1), not in the absence of LPS (curve 4), indicating that LPS was required for CD14/TLR4 receptor aggregation.

To demonstrate the sequence of LPS/CD14/TLR4 ligand-receptor complex aggregation, dynamic-emission RET was performed on live HRPE cells (Fig. 4). On exposure to LPS, early TRITC-LPS/FITC–anti-CD14 mAb RET (curve 2) was observed, whereas FITC-LPS/TRITC–anti-TLR4 mAb RET (curve 3) was observed after delays of approximately 200 seconds. In the presence of LPS, FITC–anti-CD14 and TRITC–anti-TLR4 exhibited strong RET (curve 1), which occurred after LPS/CD14 RET but before TLR4/LPS RET. CD14/TLR4 RET did not occur in the absence of LPS (curve 4). Dynamic-emission RET showed that LPS bound first to CD14 (curve 2) and induced the aggregation of CD14/TLR4 (curve 1), bringing LPS into close approximation with TLR4 (curve 3).

LPS Induction of HRPE TLR4 Gene and Protein Expression

Semiquantitative PCR (Fig. 5A) and Western blot analysis (Fig. 5B) confirmed the presence of constitutive gene expression and protein production by HRPE cells, as previously described.[37] LPS (100 ng/mL) resulted in the upregulation of *TLR4* gene expression, which was also translated to the protein level. Blocking anti-CD14 or anti-TLR4 mAb proved to be equally effective at inhibiting the LPS-induced increases in HRPE TLR4 expression. Simultaneous use of both antibodies however, did not confer additional inhibition.

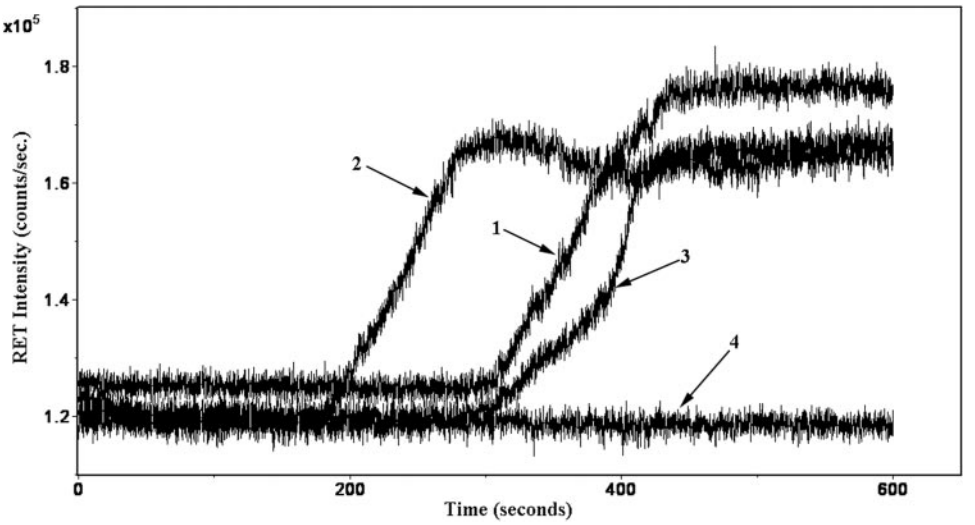

FIGURE 4. Dynamic, quantitative analysis of TLR4-CD14 receptor complex assembly caused by LPS binding. Using a photomultiplier tube detector, photon count rates were measured. Intensity was plotted at the ordinate, and time was given at the abscissa. A region surrounding one of the bound, fluorescence-labeled molecules was selected by an iris in a back focal plane of the microscope. RET fluorescence measured at 590 nm when HRPE cells were co-labeled, as indicated in Figures 1, 2, and 3, with fluorescent mAb to CD14 and TLR4 (*arrow 1*), CD14 and LPS (*arrow 2*), and TLR4 and LPS (*arrow 3*). The kinetics of the close association of the molecule pairs is shown. LPS bound first to CD14 (*arrow 2*) and was followed by CD14 and TLR4 (*arrow 1*) association and then by TLR4 and LPS (*arrow 3*) association. CD14 and TLR4 failed to exhibit RET in the absence of LPS (*arrow 4*).

LPS-Induced, TLR4-Mediated, and CD14-Mediated HRPE IL-8 Production

HRPE cells exposed to LPS (100 ng/mL) secreted substantial amounts of IL-8 (Fig. 6). Blocking anti-TLR4 mAb significantly reduced ($P = 0.018$) LPS induction of IL-8, and blocking anti-CD14 mAb was even more effective ($P < 0.001$). As expected, using both blocking mAbs also significantly inhibited LPS induction of HRPE IL-8 ($P < 0.001$) but was not significantly more effective ($P = 0.858$) than using anti-CD14 mAb alone.

Discussion

Toll is a family of eight genes whose products are pattern recognition proteins that trigger the synthesis of antimicrobial peptides in *Drosophila*.[31–33] Human homologues of *toll* (toll-like receptors [TLRs]) are relevant to adaptive immunity,[57] comprising a family of more than a dozen proteins mediating the recognition of molecules such as LPS, lipoteichoic acid, bacterial lipoprotein, zymosan, peptidoglycan, flagellin, and bacterial DNA.[31,33,34] TLRs are type 1 transmembrane proteins characterized by extracellular leucine-rich repeats and an intracellular region homologous to the internal domain of the interleukin-1 receptor.[58] Expressed by many immune cells, including neutrophils, macrophages, and lymphocytes, the cellular signaling mechanisms of TLRs and IL-1 receptors are similar and lead to cytokine expression.[32,33]

Our results indicate that HRPE cells also have functional TLR4 receptors that participate with CD14 receptors to mediate LPS binding and induction of HRPE IL-8. In this strategic location at the outer blood-retina barrier, CD14/TLR4 complexes may interact with circulating LPS or other bacterial, viral, and fungal components, resulting in chemokine elaboration by HRPE cells that may be important in ocular defense. The novel finding that LPS-HRPE CD14/TLR4 binding enhances HRPE TLR4 gene and protein expression suggests that a positive feedback loop, heightening TLR4-mediated HRPE defensive responses, occurs on continued exposure of HRPE CD14 and TLR4 to pathogens. Alternatively, however, HRPE CD14 and TLR4 binding of circulating components of infectious pathogens or endogenous ligands that may be present in various retinal diseases may be important to the development, persistence, and exacerbation of uveitis and degenerative retinal diseases because of upregulated TLR4 signaling.

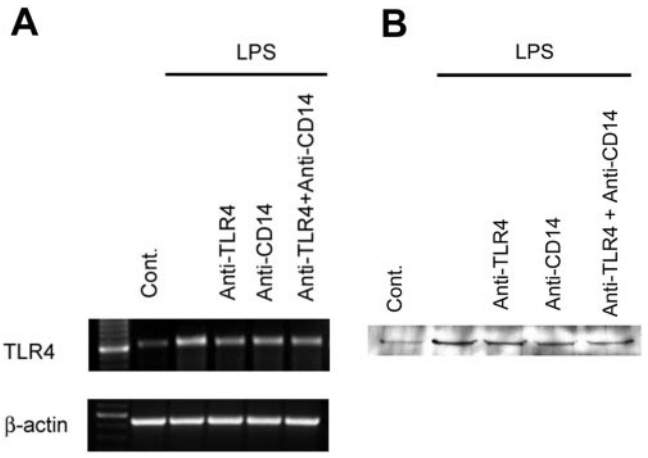

Figure 5. LPS induced TLR4 in HRPE cells. *TLR4* gene expression as measured by semiquantitative PCR (**A**) and protein production as analyzed by Western blot (**B**) were upregulated by HRPE exposure to LPS. Blocking mAb to TLR4 and CD14 inhibit LPS upregulation of TLR4 mRNA expression and protein production.

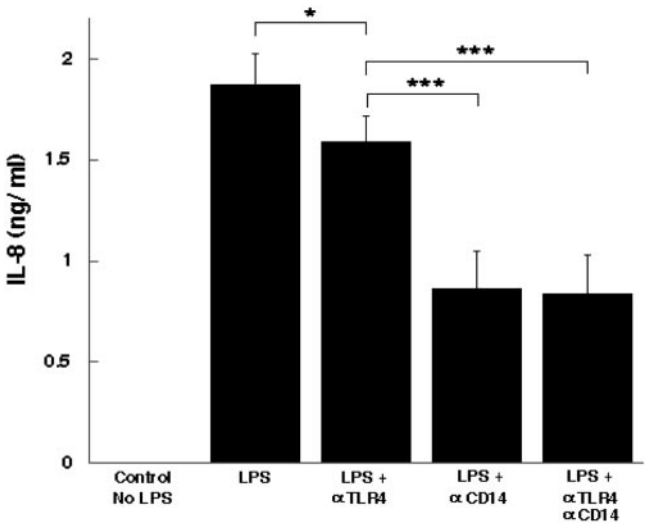

Figure 6. LPS-induced, TLR4- and CD14-mediated HRPE IL-8 production. LPS (100 ng/mL) induction of HRPE IL-8 is significantly inhibited by blocking anti-TLR4 ($P = 0.018$) or anti-CD14 ($P < 0.001$) mAb either alone or in combination ($P < 0.001$). Polymyxin B completely abrogated HRPE response to LPS (data not shown). $^{*}P < 0.05$; $^{***}P < 0.001$.

To study the interactions of LPS, TLR4, and CD14 on HRPE cells, the physical proximity of these molecules was assessed using RET imaging.[15] This format detected the proximity of the membrane-bound molecules (within 8 nm) by detecting the migration of excitation energy from donor (FITC) to acceptor (TRITC) chromophores attached to LPS and nonblocking anti-CD14 and TLR4 mAb. The physical association of these molecules, however, did not demonstrate that the LPS-receptor complex was functional. We showed that this complex on HRPE cells was functional by significantly inhibiting LPS-induced HRPE IL-8 with specific blocking anti-TLR 4 and anti-CD14 mAb (Fig. 6). These data strongly suggest that CD14/TLR4 is a functional HRPE receptor complex that binds LPS and subserves its classical role of LPS recognition, binding, and cell activation.

Steady state RET and excitation RET (Figs. 1, 2, 3) demonstrated that LPS was required for HRPE TLR4 and CD14 receptor aggregation, but dynamic RET (Fig. 4) revealed the temporal sequence of binding to these receptors, illuminating a process that has important physiologic and pathologic implications. LPS was found to bind to CD14 first, followed by CD14/TLR4 aggregation, and finally by close approximation of LPS and TLR4. This technique also confirmed that LPS was required for CD14 and TLR4 aggregation on HRPE cells. To our knowledge, this is the first time this LPS-dependent temporal sequence of CD14/TLR4 association has been shown in any cell type.

The initial binding of LPS to CD14 may explain, in part, the patterns of reduction in LPS-induced HRPE TLR4 and IL-8 expression that we observed in the presence of blocking anti-TLR4 and anti-CD14 mAb. Both antibodies appeared to be equally effective at inhibiting LPS-induced TLR4 gene and protein expression, but their simultaneous use did not further suppress this induction (Figs. 5A, 5B), suggesting that CD14 blocking of initial LPS binding was highly effective and was not further increased by blocking of the secondary receptor, TLR4. For IL-8, in addition, TLR4 blocking was less effective than CD14 blocking (Fig. 6). Although TLR4 is the primary transducer of LPS signals, the less impressive effect of TLR4 blocking may be related to the fact that LPS remains bound to CD14, which separates from TLR4 and rapidly recycles between the

plasma membrane and the Golgi apparatus.[59] This dynamic process may result in enhanced LPS signaling that is best blocked by inhibiting LPS before it binds to CD14, thereby preventing cyclical signal enhancement. TLR4 blocking might also have been less effective because HRPE cells have significant amounts of intracellular TLR4, a finding we confirmed by showing that HRPE cells permeabilized with 1% BRIJ-58 detergent display diffuse intracytoplasmic staining (results not shown) that contrasted with the delicate cell surface staining seen in Figures 1C and 2F. Thus, TLR4 blocking by mAb may be incomplete because sequestered intracytoplasmic TLR4 may replenish the cell surface with TLR4 that is not blocked.

Our findings demonstrate the ligand-dependent promiscuity of TLR4 on HRPE cells, implying dynamic HRPE responses to their environmental cues. We previously showed that TLR4 participates with CD36 in HRPE transmembrane signaling in response to photoreceptor outer segment binding.[37] In that study, outer segments first bound to HRPE CD36; this was followed by CD36/TLR4 aggregation to the endogenous retinal ligand. In this study, we observed a similar scenario of LPS binding to HRPE CD14 followed by CD14/TLR4 aggregation in response to the exogenous ligand. These findings suggest that different endogenous and exogenous ligands have the capacity to engage various combinations of HRPE receptors to subserve physiologic and pathologic processes. RPE cells are likely to avoid the release of proinflammatory cytokines when binding photoreceptor outer segments by using TLR4 clustered with coreceptors, such as CD36,[37] that are distinct from the coreceptors they use to cluster with TLR4 when binding LPS. The ligand-dependent, distinct receptor clusters trigger separate signaling pathways. Those induced by outer segments do not stimulate proinflammatory mediators, whereas those induced by LPS cause strong proinflammatory signaling, likely mediated by NF-κB[60] binding to transcription sites of IL-8 and other proinflammatory cytokines. This contention is further supported by our previous findings demonstrating CD11b and urokinase plasminogen receptor (uPAR) aggregation at the leading edges of migrating HRPE cells, permitting concentration of pericellular proteolysis in the direction of HRPE migration.[61]

In summary, TLR4 is a multifunctional receptor expressed by HRPE cells at the blood-retina barrier. HRPE TLR4 subserves the classical role of LPS binding in conjunction with CD14, mediating HRPE proinflammatory cytokine signaling known to occur in other cell types. Roles for HRPE TLR4 in mechanisms other than ocular defense from infectious agents and recognition of photoreceptor outer segments remain to be elucidated, but a recent study indicates its potential importance in the pathogenesis of age-related macular degeneration.[58]

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