TLR4 MEDIATES HUMAN RETINAL PIGMENT EPITHELIAL ENDOTOXIN BINDING AND CYTOKINE EXPRESSION

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ABSTRACT

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

Methods: Cultured human HRPE cells were examined for TLR4 expression by immunofluorescence, Western blot analysis, and reverse transcription polymerase chain reaction (RT-PCR). HRPE cells labeled with fluorescent monoclonal antibodies (mAb) 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-8 (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 ($P < .05$) in the presence of blocking mAb to TLR4 or CD14. Up-regulation of HRPE TLR4 gene and protein expression occurred in response to LPS exposure and was inhibited by mAb to TLR4 or CD14.

Conclusion: 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.

Trans Am Ophthalmol Soc 2005;103:126-137

INTRODUCTION

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 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 (Elner VM, ARVO Meeting, 1989, Abstract),[1] mannose-6-phosphate receptors (Elner VM, ARVO Meeting, 1982, Abstract),[2,3] scavenger receptors including type I/II receptors (Elner SG, ARVO Meeting, 1984, Abstract; Elner VM, ARVO Meeting, 1990, Abstract),[4] CD68,[5] and CD36.[6,7] RPE receptors mediating antigen-specific immune responses include CD16 (Fc RIII) immunoglobulin,[1] HLA-DR1,[8] and intracellular adhesion molecule-1 (ICAM-1)[5] receptors. Ambient proinflammatory cytokines, including interferon-γ, tumor necrosis factor-α, and IL-1β, up-regulate the expression of RPE HLA-DR[8] and ICAM-1[5] as well as the secretion of numerous RPE cytokines, including IL-8, an important RPE C-X-C chemokine that attracts and activates neutrophils and eosinophils (Elner VM, ARVO Meeting, 1990, Abstract).[9] The RPE expression of these receptors and potent proinflammatory cytokines, including IL-8, implies important roles for RPE cells in ocular defense.

Among the immune receptors expressed are CD14 (Elner VM, ARVO Meeting, 1989, Abstract)[10] and TLR4.[11] CD14, the primary receptor for lipopolysaccharide (LPS), is a 55-kDa glycosylphosphatidylinositol–anchored glycoprotein initially identified on the surface of mononuclear phagocytes and neutrophils.[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 toll-like receptor 4 (TLR4).[15,16] LPS binding induces transmembrane signals[17,18] leading to nuclear factor–κB[19,20] activation that results in cytokine production, including IL-8.[10,21-23]

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]

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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 a pattern recognition receptor, which binds 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, as well as 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[10] 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 as well as the effect of LPS on HRPE TLR4 expression. TLR4 HRPE expression, in conjunction with CD14 expression at the blood-retina barrier, may play roles in ocular defense and other pathophysiologic mechanisms.

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 Helsinki agreement.[38] In brief, the sensory retina was separated gently from the HRPE monolayer, and the HRPE cells were removed from Bruch's membrane by 1-hour incubation with papain (5 μg/mL) (Sigma Chemical Co, St Louis, Missouri). Isolated HRPE cells were seeded into Falcon Primaria flasks (Becton-Dickinson Inc, Lincoln Park, New Jersey) 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). The cultured HRPE cells formed monolayers showing typical polygonal morphology and pigmentation of scattered cells and exhibited uniform immunohistochemical staining for cytokeratin characteristic for these epithelial cells.[39] In all experiments, simultaneous, parallel assays were performed on second to fourth passaged 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 immunofluorescent staining, TLR4 semiquantitative PCR, TLR4 Western blot analysis, or cytokine enzyme-linked immunosorbent assay (ELISA).

HRPE CELL STIMULATION WITH ENDOTOXIN (LPS)

Before experiments, HRPE cells were incubated in serum-free medium for 24 hours. HRPE cells were then incubated in DMEM, or in DMEM containing 100 ng/mL of 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 media were collected and then centrifuged to remove particulates. Conditioned media were stored at –70°C until ELISA was performed.

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

FLUORESCENT DEMONSTRATION OF LPS, TLR-4, AND CD14 ASSOCIATION ON HRPE CELLS

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

For fluorescent studies, mouse anti-human CD14 (clone CRIS-6) and anti-human TLR4 (clone HTA1216) mAb were obtained from BioSource International (Camarillo, California) and K. 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] The fluorescent conjugates were separated from unreacted fluorochromes by Sephadex G-25 (Sigma) column chromatography. Purified conjugates were dialyzed against PBS at pH 7.4 overnight at 4°C.

HRPE cells, grown to confluency 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 media 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. The 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 axiovert-inverted fluorescence microscope with HBO-100 mercury illumination (Carl Zeiss, New York, New York) interfaced

to a Dell 410 workstation via Scion SG-7 video card (Vay Tek, Fairfield, Iowa) was employed for cell examination. The fluorescence images were collected by an intensified charge-coupled device camera, model XC-77 (Hamamatsu, Hamamatsu City, Japan) and processed with Scion Image Software. 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, Vermont). Long-pass dichroic mirrors at 510 and 560 nm were used for FITC and TRITC, respectively. For resonance energy transfer (RET) microscopy,[51] the 485DF20 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 by using Zeiss polarizers and a charge-coupled device camera (Dage-MTI, Michigan City, Indiana). Background-subtracted digitized images were averaged and then electronically stored.

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

SEMIQUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) 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′; and β-actin, 5′-GTGGGGCGCCCCAGGCACCA-3″ and 5′-CTCCTTAATGTCACGCACGATTTC-3′. Total RNA was extracted by using TRIzol reagent (GIBCO BRL), 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 a 1-minute denaturation at 94°C, a 1-minute primer annealing at 55°C, and a 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 orthovanadage, 10 mM sodium pyrophosphate, 1mM 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 18361xg for 15 minutes at 4°C.

Western blot analyses of cellular extracts from HRPE cells followed the manufacturer's procedure. Briefly, samples containing 20 mg of proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then were electrotransferred to nitrocellulose membranes. For signal protein detection, samples were blocked with a solution of Tris-buffered saline containing 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. The membranes were incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature and washed three times with TBST. The membranes were then visualized by using an enhanced chemiluminescent technique.

ENZYME-LINKED IMMUNOABSORBENT ASSAY FOR IL-8

Antigenic IL-8 was quantitated by using a double-ligand ELISA method, as described previously.[55] Briefly, ELISA was performed on serial dilutions of HRPE conditioned media. Conditioned media were aspirated from the surface 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, Minnesota). This ELISA method consistently detected IL-8 concentrations of more than 10 pg/mL. Standards included 0.5 log dilutions of rhIL-8 (R&D Systems) from 5 pg to 100 ng per well.

STATISTICAL ANALYSIS

Individual experiments were performed on three different HRPE cell lines. Each cell line displayed similar increases or decreases over control levels. Data are expressed as mean ± standard deviation. Various assay conditions were evaluated by using analysis of variance test with a post hoc analysis (Schiff multiple comparison test); *P* values <.05 were considered to be 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 (Figures 1A, 2A, 2E). HRPE cells labeled with TRITC-anti-TLR4 mAb, but not labeled idiotypic mAb, demonstrated delicate staining, which was enhanced along intracellular interfaces (Figures 1C, 2C, 2G), confirming our previous observations of HRPE TLR4 expression.[37] Simultaneous FITC-LPS labeling of HRPE cultures demonstrated discrete cell surface binding (Figure 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 (Figure 1D) yielded photochemical confirmation of the extremely close 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 (Figure 2). Under both conditions, discrete immunolabeling of live HRPE cells revealed similar discrete localization of CD14 (Figures 2B, 2F) and TLR4 (Figures 2C, 2G), suggesting that endotoxin was 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).

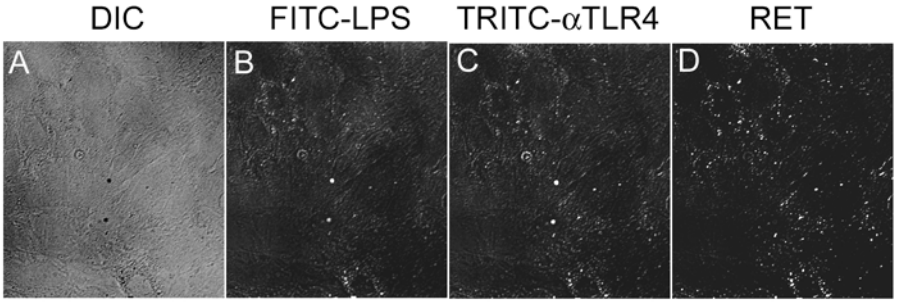

FIGURE 1

Fluorescent imaging studies demonstrate TLR4 and LPS co-localization on live HRPE cells. A, Differential image contrast (DIC) micrograph of cultured HRPE cells in confluent cell cultures. B, Cell surface binding of FITC-labeled LPS. C, Cell surface fluorescence due to bound TRITC-labeled anti-(α)-TLR4 in the presence of bound FITC-labeled LPS. The pattern of TLR4 labeling is similar in distribution to that observed for LPS binding. D, Resonance energy transfer (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.

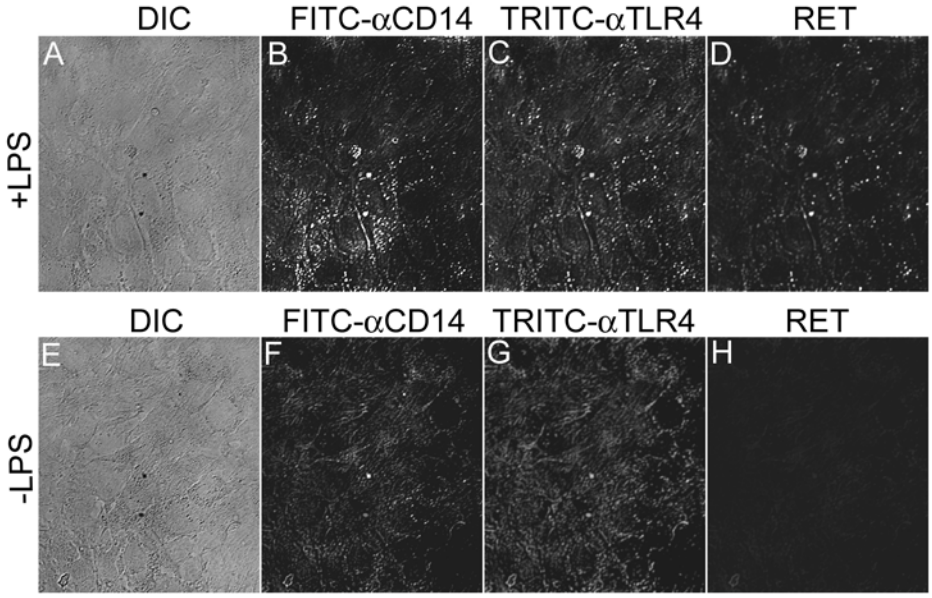

FIGURE 2

Fluorescent imaging studies demonstrate TLR4 and CD14 co-localization on live HRPE cells. A and E, Differential image contrast (DIC) micrographs of cultured HRPE cells in confluent cell cultures; B and F, Cell surface binding of FITC-labeled anti-CD14; C and G, Cell surface fluorescence due to bound TRITC-labeled anti-(α)-TLR4 in the presence of bound FITC-labeled α-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 and H, Resonance energy transfer (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).

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 (Figure 3). Peak excitation of FITC at 488 nm (measured in abscissa in Figure 3) resulted in peak fluorescence at 590 nm (measured in ordinate in Figure 3) by the HRPE cultures also labeled with TRITC only when the molecule pairs labeled with FITC and TRITC were in extremely close proximity to within 7 nm of each other, thereby permitting 488-nm-induced FITC emission at 520 nm to excite TRITC and 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 observed only in the presence of LPS (curve 1), and not in the absence of LPS (curve 4), indicating that LPS was required for CD14/TLR4 receptor aggregation.

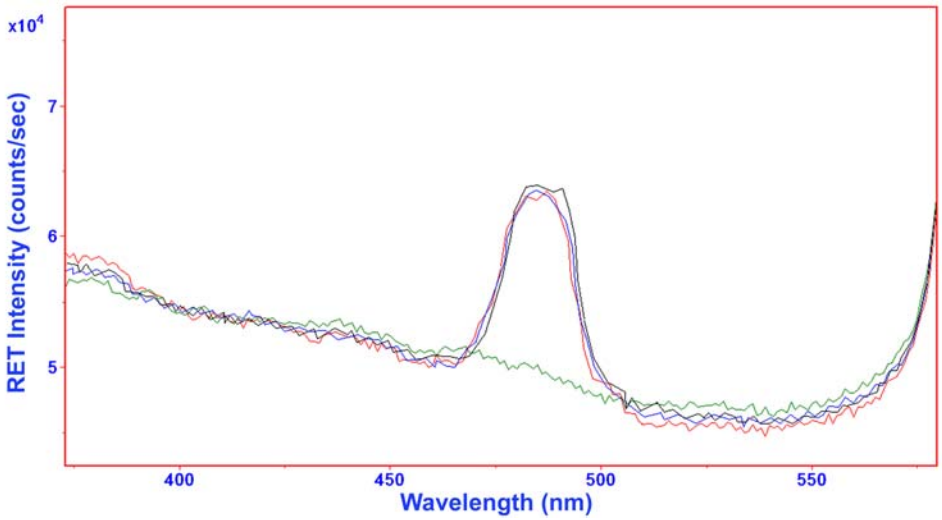

FIGURE 3

Resonance energy transfer (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 colabeled, as indicated in Figures 1 and 2, with fluorescent mAb to CD14 and TLR4 (black), CD14 and LPS (red), and TLR4 and LPS (blue). RET indicates simultaneous, close approximation of all three molecule pairs at steady state due to 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 (green).

To demonstrate the sequence of LPS/CD14/TLR4 ligand-receptor complex aggregation, dynamic emission RET was performed on live HRPE cells (Figure 4). Upon exposure to LPS, early TRITC-LPS/FITC-anti-CD14 mAb RET was observed (curve 2) while FITC-LPS/TRITC-anti-TLR4 mAb RET (curve 3) occurred 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). Taken together, the dynamic emission RET shows that LPS binds first to CD14 (curve 2) and induces 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 (Figure 5A) and Western blot analysis (Figure 5B) confirmed the presence of constitutive gene expression and protein production by HRPE cells as previously described.[37] LPS (100 ng/mL) resulted in up-regulation 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 did not confer additional inhibition, however.

LPS-INDUCED, TLR4- AND CD14-MEDIATED HRPE IL-8 PRODUCTION

HRPE cells exposed to LPS (100 ng/mL) secreted substantial amounts of IL-8 (Figure 6). Blocking anti-TLR4 mAb significantly reduced ($P = .018$) LPS induction of IL-8, whereas blocking anti-CD14 mAb was even more effective ($P < .001$). As expected, using both blocking mAb also significantly inhibited LPS induction of HRPE IL-8 ($P < .001$) but was not significantly more effective ($P = .858$) than using anti-CD14 mAb alone.

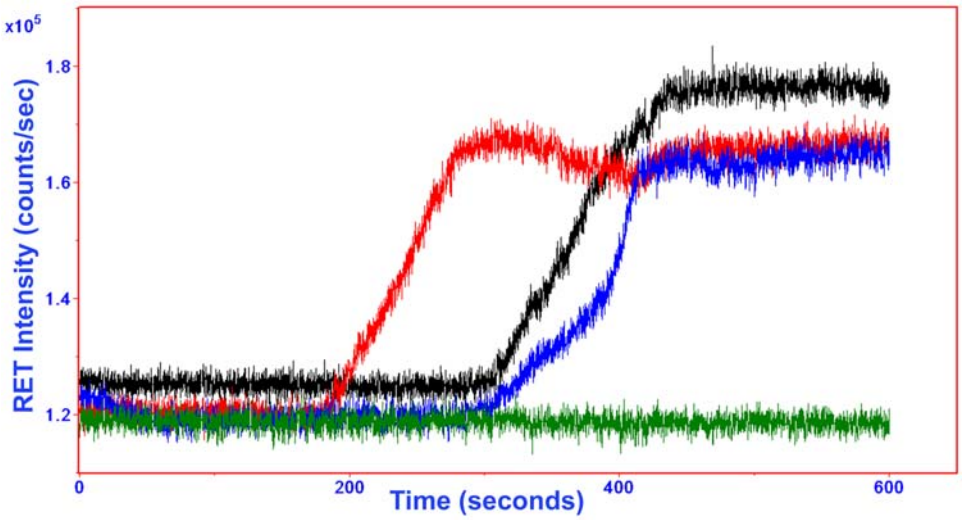

FIGURE 4

Dynamic, quantitative analysis of TLR4-CD14 receptor complex assembly due to LPS binding. Photon count rates were measured with a photomultipler tube detector. Intensity was plotted at the ordinate, and time was given at the abscissa. A region surrounding one of the bound, fluorescently labeled molecules was selected by an iris in a back focal plane of the microscope. Resonance energy transfer (RET) fluorescence measured at 590 nm when HRPE cells are colabeled, as indicated in Figures 1, 2, and 3, with fluorescent mAb to CD14 and TLR4 (black), CD14 and LPS (red), and TLR4 and LPS (blue). The kinetics of the close association of the molecule pairs is shown. LPS binds to CD14 (Figure 2) followed in time by CD14 and TLR4 association, and subsequent TLR4 and LPS association. CD14 and TLR4 fail to exhibit RET in the absence of LPS (green).

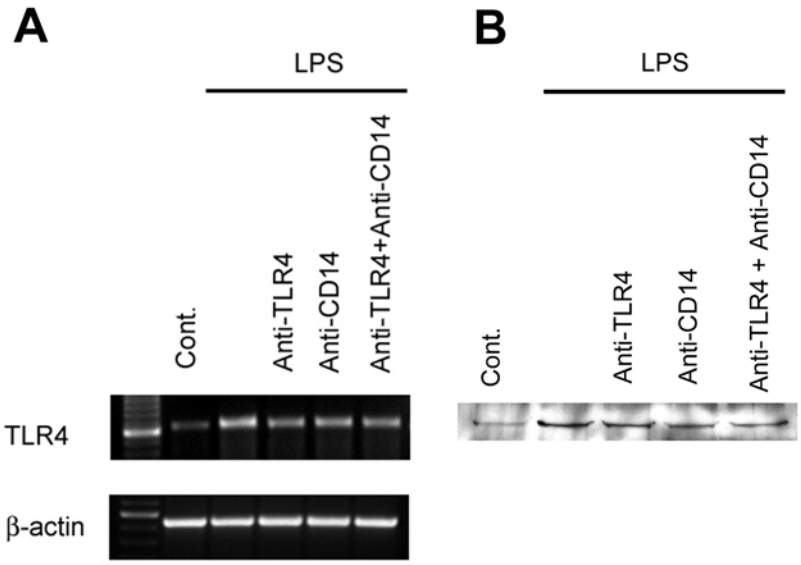

FIGURE 5

LPS induces TLR4 in HRPE cells. TLR4 gene expression as measured by semiquantitative PCR (A) and protein production as analyzed by Western blot (B) are up-regulated by HRPE exposure to LPS. Blocking mAb to TLR4 and CD14 inhibit LPS up-regulation of TLR4 mRNA expression and protein production.

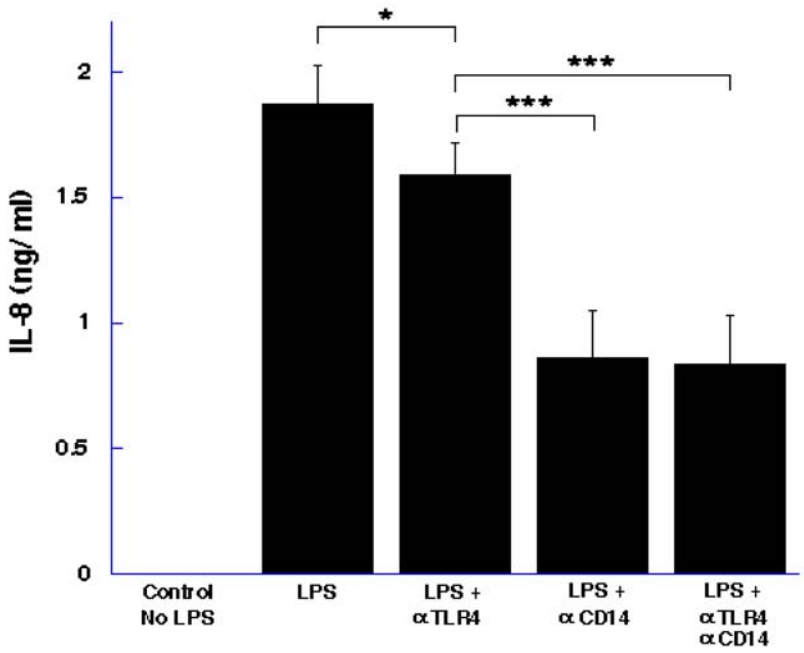

FIGURE 6

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-(α)-TLR4 (P = .018) or α-CD14 (P < .001) mAb either alone or in combination (P < .001). Polymyxin B completely abrogated HRPE response to LPS (data not shown). *P < .05, ***P < .001.

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 adaptive immunity,[57] comprising a family of over 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 I 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 possess 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 upon 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 due to up-regulated TLR4 signaling.

To study the interactions of LPS, TLR4, and CD14 on HRPE cells, the physical proximity of these molecules was assessed by using RET imaging.[15] This format detected the extremely close 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 (Figure 6). These data strongly suggest that CD14/TLR4 is a functional HRPE receptor complex that binds LPS and subserves its classic role of LPS recognition, binding, and cell activation.

Steady-state RET and excitation RET (Figures 1 through 3) demonstrated that LPS was required for HRPE TLR4 and CD14 receptor aggregation, but dynamic RET (Figure 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 (Figures 5A and B), suggesting that CD14 blocking initial LPS binding was highly effective and not further increased by blocking the secondary receptor, TLR4. For IL-8, in addition to the above, TLR4 blocking was less effective than CD14 blocking (Figure 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.

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 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. This contention is further supported by our previous findings demonstrating CD11b and urokinase plasminogen activator receptor aggregation at the leading edges of migrating HRPE cells, permitting concentration of pericellular proteolysis in the direction of HRPE migration.[60]

In summary, TLR4 is a multifunctional receptor expressed by HRPE cells at the blood-retina barrier. HRPE TLR4 subserves the classic 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.[61]

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PEER DISCUSSION

DR ROBERT N. FRANK. A search of PubMed yields 2,529 entries for the key word "Toll-like receptor," but a search using the terms "Toll-like receptor and eye," or "Toll-like receptor and eye diseases" yields only 19 entries, plus an additional one for the recently published (on the Internet prior to print publication) paper by Zareparsi and associates,[1] which is the last reference cited in the present manuscript by Dr Susan Elner and her colleagues. Clearly, this important, and recently described (in 1997-98)[2] class of membrane receptors has until now received little attention from ophthalmologists and vision researchers.

What are the Toll-like receptors, and what might their physiological (or pathophysiological) roles be in ocular tissues, and in particular, as Dr Elner describes here, in the retinal pigment epithelium? Toll is a protein that was first described in the embryo of the fruit fly Drosophila that governs dorsal-ventral body patterning,3 but also functions in the immune response.4 The Toll-like receptors (TLRs) of vertebrates, which are homologous to Drosophila Toll, are a family of ten cell receptor proteins that have important roles in the adaptive immune response, most importantly in the recognition of surface molecules in harmful invaders, such as bacterial lipopolysaccharide (endotoxin). Toll-like receptors have been described in several tissues of the eye, including cornea, uveal tract, retina, sclera, and conjunctiva. Kumar and associates5 found genes for TLRs 1-7, 9, and 10 in retinal pigment epithelial cells with TLRs 1 and 3 the most highly expressed. Kumar and associates also found the proteins for TLRs 2-4. Chang and associates6 reported TLR4 and its co-receptor CD14 mRNAs throughout the human uvea, retina, sclera and conjunctiva. However, in apparent contrast to the results of Dr Elner and her collaborators, they found the respective proteins solely in antigen-presenting dendritic cells. Toll-like receptors not only stimulate adaptive immunity in response to exogenous pathogens but, as the present authors showed in a previous paper, they signal to the retinal pigment epithelial cell the presence of photoreceptor outer segment tips from the same species for phagocytosis, while rejecting outer segment tips from other species.7 Finally, in an intriguing and very recent paper from the authors' own institution, Zareparsi and associates1 reported an increased risk of age-related macular degeneration in patients with the G allele at TLR4 residue 299. The possible association of a mutation in the TLR4 gene with age-related macular degeneration is an obviously important topic for further exploration.

In the present paper, Dr Susan Elner and colleagues have demonstrated that the concerted action of TLR4 and CD14 following stimulation by LPS. CD14 requires a co-receptor, such as TLR4, because the CD14 molecule does not extend through the cell membrane and therefore cannot initiate an intracellular signaling cascade without the assistance of another receptor, such as TLR4, which does have an intracellular domain. The end result of transmembrane signaling by this receptor cascade is the release of inflammatory cytokines, such as interleukin-8.[8] This response has been reported in several tissues, of which the retinal pigment epithelium is one. One novel result that is demonstrated in this presentation is the temporal sequence in which, Dr. Elner and her associates show, lipopolysaccharide binds first to CD14, which is followed by CD14 association with TLR4 and then by association of

lipopolysaccharide with TLR4.

I have a number of questions about these results. The authors state that fluorescently labeled TLR4, lipopolysaccharide, and CD14 co-localize along intracellular cell membrane interfaces. At the magnifications shown in their photomicrographs, I cannot distinguish this localization. Additionally, more extensive immunolabeling experiments would be helpful to learn whether these molecules label the retinal pigment epithelial cell surface very widely, or whether they are preferentially localized to one surface, say, the apical (as one might expect from the role of TLR4 in the phagocytosis of photoreceptor outer segments), or the basal or basolateral (as one might expect if their major role is triggering an immune response when they are stimulated by a bacterial, or other, pathogen). It's not clear to me how the resonance energy transfer experiments localize the CD14 and TLR4 sites to within 8 nm of each other. How precise is this localization? Finally, and in view of the current emphasis on "translational" research that leads directly from discoveries in the laboratory to clinical benefits for patients, further exploration of the possible roles of TLR4 and other TLRs in the retinal pigment epithelium in ocular disease will clearly be important. It may be possible, for example, to produce knockouts, transgenics, or other mutations in TLR molecules specifically localized to the retinal pigment epithelium using Cre-Lox methodology and specific promoters, and by using tetracycline to "turn on" or "turn off" the specific genes in mutant mice and then to learn how the mutation affects the phenotype. Does the TLR4/CD14 system have a role in uveitis? In infectious disorders of the retina/choroid/retinal pigment epithelium? In age-related macular degeneration, as suggested by the recent paper of Zareparsi and associates? How might TLR4 distinguish pathogenic stimuli, such as bacterial lipopolysaccharide, from physiological stimuli such as photoreceptor outer segments? What prevents activation of TLR4 by physiological stimuli from causing an inflammatory reaction, such as production of interleukin-8 and other inflammatory cytokines? One possible explanation of a role for a mutated TLR-4 gene in age-related macular degeneration, especially in view of the current interest in inflammation as a pathogenic factor in this disease, might be that the mutation alters signaling pathways, so that the TLR-4 receptor interprets the photoreceptor outer segment membrane as an inflammatory stimulus.

As with many excellent experiments, the results presented by Dr Elner and her colleagues lead to many more questions, the answers to which will greatly expand our knowledge of the functions of the RPE in health and disease. Dr Elner and her colleagues have taken a pioneering first step in the study of Toll-like receptors in the retinal pigment epithelium. We can anticipate more exciting results from them in the future.

ACKNOWLEDGMENTS

I thank Linda Hazlett, Ph.D., Department of Anatomy and Cell Biology and Fu-Shin Yu, Ph.D., Department of Ophthalmology, both at Wayne State University School of Medicine, for helpful discussions in the preparation of this discussion.

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DR CHI-CHAO CHAN. Have you looked at other molecules in the TLR4 signal transduction pathway from TLR4 to NF kappa B?

DR SUSAN G. ELNER. In response to Dr Chan's question: yes, we plan to look at the intracellular pathways involved in TLR4, CD14, and LPS signaling in retinal pigment epithelial cells.

Dr Frank, I appreciate your additional review of TLR4 since my presentation was limited in time. How does RPE TLR4 and CD14 distinguish between pathologic LPS recognition and physiologic outer segment recognition? It is possible that TLR4 pairs up with different RPE cell surface receptors other than CD14, depending upon the ligand involved. With LPS recognition by RPE cells, both CD14 and TLR4 are involved, as shown in this study. In contrast, our previous work on the binding of photoreceptor outer segments by RPE cells showed an association of CD36 with TLR4, indicating TLR4 and CD36 are involved in the recognition of the outer segments[1]. We did not specifically look at CD14 in that study, so we do not know if the CD14 is involved along with TLR4 in the outer segment recognition. The pairing of TLR4 with different receptors may help in RPE recognition of different molecules and result in different intracellular signaling and RPE responses.

How does a mutated TLR4 function in diseases such as macular degeneration? Your suggestions of perhaps the binding of outer

segments to mutant TLR to induce an alternate inflammatory response is a reasonable hypothesis since we know that macrophages and low-grade inflammation are increasingly recognized as having a role in macular degeneration. We would like to investigate whether MCP-1, which is a pro-inflammatory chemokine that has been recognized in macular degeneration, is important. These are future lines of study.

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