

Toll-Like Receptor 4 (TLR4) of Retinal Pigment Epithelial Cells Participates in Transmembrane Signaling in Response to Photoreceptor Outer Segments

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ABSTRACT Retinal pigment epithelial (RPE) cells mediate the recognition and clearance of effete photoreceptor outer segments (POS), a process central to the maintenance of normal vision. Given the emerging importance of Toll-like receptors (TLRs) in transmembrane signaling in response to invading pathogens as well as endogenous substances, we hypothesized that TLRs are associated with RPE cell management of POS. TLR4 clusters on human RPE cells in response to human, but not bovine, POS. However, TLR4 clustering could be inhibited by saturating concentrations of an inhibitory anti-TLR4 mAb. Furthermore, human POS binding to human RPE cells elicited transmembrane metabolic and calcium signals within RPE cells, which could be blocked by saturating doses of an inhibitory anti-TLR4 mAb. However, the heterologous combination of bovine POS and human RPE did not trigger these signals. The pattern recognition receptor CD36 collected at the POS–RPE cell interface for both homologous and heterologous samples, but human TLR4 only collected at the human POS–human RPE cell interface. Kinetic experiments of human POS binding to human RPE cells revealed that CD36 arrives at the POS–RPE interface followed by TLR4 accumulation within 2 min. Metabolic and calcium signals immediately follow. Similarly, the production of reactive oxygen metabolites (ROMs) was observed for the homologous human system, but not the heterologous bovine POS–human RPE cell system. As (a) the bovine POS/human RPE combination did not elicit TLR4 accumulation, RPE signaling, or ROM release, (b) TLR4 arrives at the POS–RPE cell interface just before signaling, (c) TLR4 blockade with an inhibitory anti-TLR4 mAb inhibited TLR4 clustering, signaling, and ROM release in the human POS–human RPE system, and (d) TLR4 demonstrates similar clustering and signaling responses to POS in confluent RPE monolayers, we suggest that TLR4 of RPE cells participates in transmembrane signaling events that contribute to the management of human POS.

KEY WORDS: RPE activation • metabolism • photoreceptor outer segments • Toll-like receptors • reactive oxygen metabolites

INTRODUCTION

Toll is a family of eight genes whose products are pattern recognition proteins that contribute to establishing the dorsal–ventral axis during embryogenesis and trigger the synthesis of antimicrobial peptides in *Drosophila* (Akira, 2000; Schuster and Nelson, 2000; Dobrovolskaia and Vogel, 2002). Janeway's group first cloned human homologues of toll (Toll-like receptors [TLRs]) and demonstrated their relevance to adaptive immunity (Medzhitov et al., 1997). Human TLRs represent a family of over a dozen proteins mediating the recognition of molecules such as lipopolysaccharide (LPS), lipoteichoic acid, bacterial lipoprotein, zymosan, peptidoglycan, flagellin, and bacterial DNA (Akira, 2000; Dobrovolskaia

and Vogel, 2002; Barton and Medzhitov, 2002). 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. They are expressed by many immune cells including neutrophils, macrophages, monocytes, mast cells, dendritic cells, T cells, and B cells (e.g., Schuster and Nelson, 2000; Termeer et al., 2002; Dobrovolskaia and Vogel, 2002). The cellular signaling mechanisms of TLRs and IL-1 receptors are similar and lead to changes in cytokine expression. Although a great deal of interest has been focused on the role of TLRs in leukocyte responses to microbes and their by-products, TLRs may be more broadly used in metazoans. Recent work has shown that TLRs are expressed

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Abbreviations used in this paper: LPS, lipopolysaccharide; mnd, motor neuron degeneration; POS, photoreceptor outer segments; RET, resonance energy transfer; ROM, reactive oxygen metabolite; RPE, retinal pigment epithelial; TLR, Toll-like receptor.

by several cell types, including leukocytes, endothelial cells, intestinal epithelial cells, corneal cells, and others (e.g., Song et al., 2001; Hornef et al., 2002). Furthermore, TLR expression has been identified in several types of tissues (e.g., heart, brain, placenta, ovary, prostate, muscle, etc.) (Schuster and Nelson, 2000).

In addition to neutrophils and monocytes/macrophages, retinal pigment epithelial (RPE) cells are also "professional" phagocytes. RPE cells lay between the choroid and photoreceptor cells of the neurosensory retina. The photoreceptors must constantly shed aged or damaged portions of their outer segments (POS). Several proteins may participate in the clearance of POS. MERTK, which is also known as the proto-oncogene *c-mer*, is a key participant in the phagocytosis of POS, as illustrated by genetic studies (D'Cruz et al., 2000; Gal et al., 2000). In addition, the pattern recognition receptor CD36 and the integrin $\alpha_v\beta_5$ have also been suggested to participate in POS clearance (Finnemann et al., 1997; Finnemann and Silverstein, 2001). As leukocyte TLRs appear to play key roles in signaling but not phagocytosis, we postulated that TLRs participate in the handling of POS by RPE cells. In the present study we show that TLR4 molecules expressed at the surface of human RPE cells participate in the cellular handling of human POS.

MATERIALS AND METHODS

Reagents and Antibodies

FITC, TRITC, and indo-1 were obtained from Molecular Probes. Anti-CD36 was obtained from PharMingen. Anti-TLR4 was a gift of K. Miyake (Saga Medical School, Saga, Japan) (Shimazu et al., 1999). FITC or TRITC-conjugated antibodies were prepared as previously described (Kindzelskii et al., 1997).

RPE Cell and POS Preparation

Human RPE cells were isolated from donor eyes as previously described (Elner et al., 1991). Primary, first, or second passaged human RPE cells were trypsinized and then seeded onto glass coverslips 5 d before experiments. Bovine and human eyes were obtained, neural retina were removed, and then POS were obtained from retinal homogenates by sucrose density gradient centrifugation (Uhl et al., 1987). POS were fluorescently labeled with FITC by the method of McLaren et al. (1993). For experimental use, a stock POS solution at 5×10^6 particles/ml was diluted to give a 10:1 ratio of POS to cells.

RT-PCR

Synthetic oligonucleotide primers based on the cDNA sequences of human TLR4 and β -actin were prepared: TLR4, 5'-TCCCTC-CAGGTTCTTGATTACAGTC-3' and 5'-TGCTCAGAACTGC-CAGGTCTG-3'; and β -actin, 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAATGTACAGCAGGATTC-3'. Total RNA was extracted by using TRIzol reagent (GIBCO BRL), according to the manufacturer's procedure. 1 μ g of RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). The cDNA was denatured for 5 min at 94°C, followed by 28 PCR cycles. Each cycle included a 1-min de-

naturation at 94°C, a 1-min primer annealing at 55°C, and a 2-min polymerization at 72°C. Each RT-PCR reaction mixture was analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Western Blot Analysis

RPE cells were lysed with lysing 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 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Lysates were incubated on ice for 15 min with shaking. The extracts were then centrifuged at 15,000 rpm for 15 min at 4°C.

Western blot analyses of RPE cellular extracts followed the manufacturer's procedure. In brief, samples containing 20 μ g of protein were separated by SDS-PAGE and then electrotransferred to nitrocellulose membranes. For 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 h, probed with anti-TLR4 mAb, and washed three times in TBST. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and washed three times with TBST. The membranes were then visualized using an enhanced chemiluminescence technique.

Immunofluorescence Staining

RPE cells were grown on glass coverslips to form subconfluent or confluent cultures. Samples were labeled using direct immunofluorescence. Cells were labeled with 80 ng/ml anti-TLR4 clone HTA1216 for 20 min at 37°C followed by four rinses with HBSS. In experiments using anti-TLR4 clone HTA125 for blocking, cells were first labeled with anti-TLR4 clone HTA1216 at 80 ng/ml. Cells were also labeled with anti-CD36 mAb at 50 ng/ml for 20 min at 37°C followed by extensive washing with HBSS at room temperature.

Fluorescence Microscopy

Cells were observed using an Axiovert fluorescence microscope (Carl Zeiss MicroImaging, Inc.) with mercury illumination interfaced to a computer using Scion image processing software (Kindzelskii et al., 1998). A narrow bandpass discriminating filter set (Omega Optical) was used with excitation at 485/22 nm and emission at 530/30 nm for FITC, and an excitation of 540/20 nm and emission at 590/30 nm for TRITC. Long-pass dichroic mirrors of 510 and 560 nm were used for FITC and TRITC, respectively. For resonance energy transfer (RET) imaging, a 485/22 nm narrow bandpass discriminating filter for excitation, and a 590/30 nm filter was used for emission (Kindzelskii et al., 1997). The fluorescence images were collected with a Peltier-cooled intensified charge-coupled device camera (Princeton Inst.).

Quantitative microfluorometry was used to evaluate RET levels. This was performed using a cooled high-sensitivity photomultiplier tube in a D104 detection system (Photon Technology International, Inc.) attached to a Carl Zeiss MicroImaging, Inc. microscope (Kindzelskii et al., 2002; Olsen et al., 2003; Petty, 2003).

Detection of NAD(P)H Oscillations and Calcium Spikes

NAD(P)H autofluorescence oscillations were detected as previously described (Kindzelskii et al., 2002; Olsen et al., 2003; Petty, 2003). To detect calcium signals, the calcium reporter indo-1 was used as previously described (Kindzelskii and Petty, 2003). An iris diaphragm was adjusted to exclude light from neighboring cells. For detection of NAD(P)H and indo-1 fluorescence, kinetic stud-

ies were performed using the quantitative microfluorometry apparatus described in the preceding paragraph. Data were analyzed using Felix software (Photon Tech. Intl.).

Reactive Oxygen Metabolites

Pericellular reactive oxygen metabolite (ROM) release from single cells was monitored as previously described (Kindzelskii et al., 1998, 2002). In brief, cells were surrounded in 2% gelatin containing 100 ng/ml dihydrotetramethylrosamine (H₂TMRos) (Molecular Probes). ROMs, especially H₂O₂, released by the cells entered the gelatin matrix, where they oxidized H₂TMRos to tetramethylrosamine (TMRos), which was detected by fluorescence microscopy.

Statistics

Comparisons between groups were performed using Student's *t* test. Data are expressed as mean ± SEM. *n* is the number of separate experiments performed. All experiments were repeated on at least three independent occasions each consisting of 20–50 separate cellular measurements. All experiments used at least three different RPE donors.

RESULTS

RPE Cells Express TLR4

We first tested the hypothesis that RPE cells express TLR4. TLR4 message was demonstrated in RPE cells following RNA extraction and RT-PCR, as shown in the top panel of Fig. 1. To demonstrate that this message yielded an antigenically intact protein, RPE cells were solubilized in Triton X-100/HEPES buffer followed by SDS-PAGE. When nitrocellulose blots were probed with anti-TLR4, protein expression was confirmed (Fig. 1, bottom). To demonstrate that TLR4 was expressed on the cell surface, living RPE cells were stained with fluo-

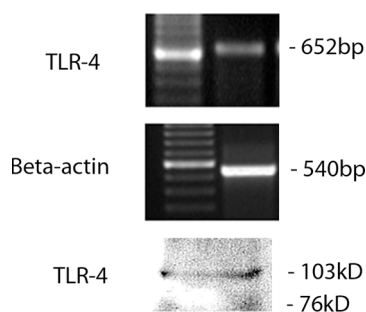


FIGURE 1. Expression of TLR4 by human RPE cells. (A) RT-PCR demonstrates the presence of TLR4 message. (B) Western blots demonstrate the presence of TLR4 antigen in RPE cells.

rescein-conjugated anti-TLR4 mAb. Direct immunofluorescence microscopy demonstrated that anti-TLR4 mAb (Fig. 2, C and G, and Fig. 3, C, G, and K), but not isotype-matched controls (not depicted), labeled RPE cells. Therefore, the TLR4 gene is expressed by RPE cells, and the protein product traffics to the cell surface.

TLR4 Clustering Accompanies POS Binding to RPE Cells, But Is Blocked by an Inhibitory Anti-TLR4 mAb

TLRs elicit transmembrane signals that participate in the clearance of pathogens and endogenous molecules by several cell types. Therefore, we tested the hypothesis that TLR4 participates in POS binding and/or signaling on RPE cells. Human RPE cells were grown until they formed a confluent monolayer on glass coverslips. Cell surface TLR4 molecules were trace labeled using a saturating dose (80 ng/ml) of rhodamine-conjugated

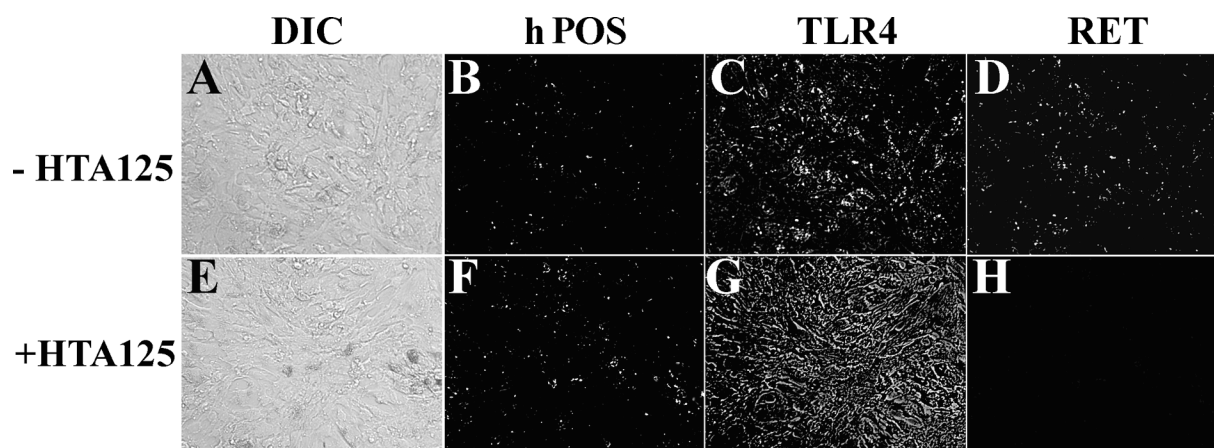


FIGURE 2. TLR4 clusters at the sites of POS binding. Representative DIC (A and E), fluorescence (B, C and F, G), and RET (D and H) micrographs are shown. RPE cells were trace labeled with TRITC-conjugated anti-TLR4 (clone HTA1216) at 80 ng/ml and then incubated for 2 h at 37°C. In panels E–H, cells were trace labeled using anti-TLR4 clone HTA1216 and then incubated with anti-TLR4 clone HTA125 at 30 µg/ml for 2 h at 37°C to block TLR4. RPE cells were then incubated with FITC-conjugated human POS, which were found to randomly label the surface of RPE cells (B and F). In the absence of anti-TLR4 clone HTA125, TLR4 clustered at sites of POS binding (C), and the anti-TLR4 clone HTA1216 mAb was found in close proximity with the POS (D). However, incubation with a saturating dose of the anti-TLR4 clone HTA125 blocked clustering of the anti-TLR4 clone HTA1216 (G) and RET with POS (H) (×375) (*n* = 3).

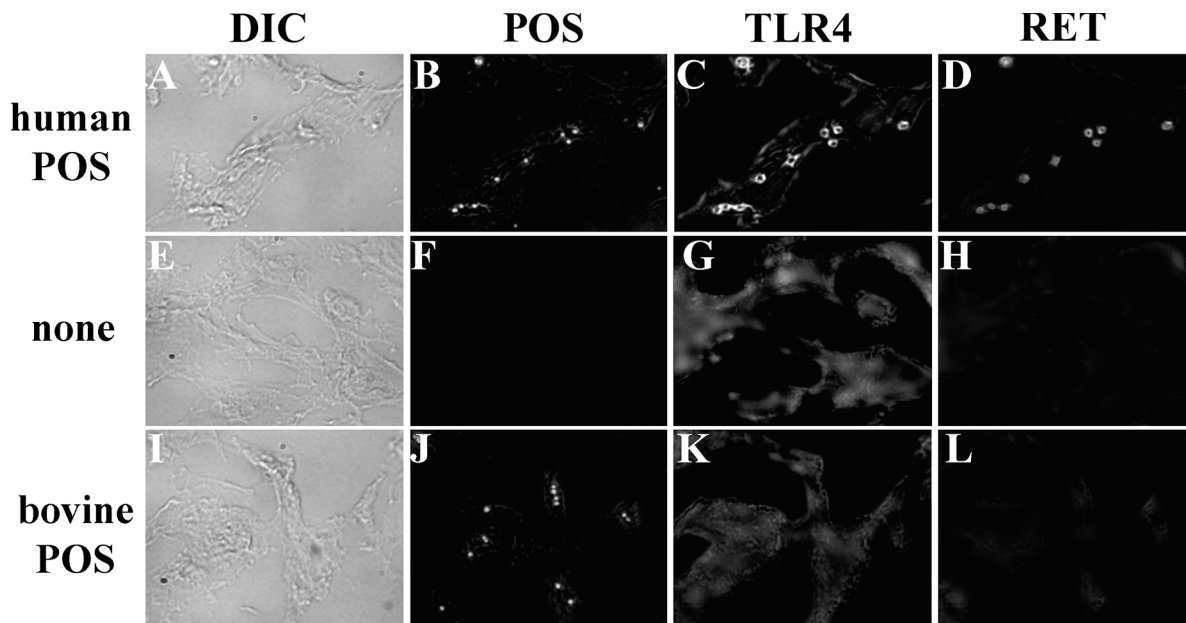


FIGURE 3. TLR4 accumulates at sites of human but not bovine POS on human RPE cells. Representative DIC (column 1), fluorescence (columns 2 and 3), and RET (column 4) micrographs are shown. RPE cells were labeled with TRITC-conjugated anti-TLR4 (clone HTA1216). Human RPE cells were incubated with FITC-conjugated human POS (A–D), no POS (E–H), or FITC-conjugated bovine POS (I–L). In the absence of POS, TLR4 was uniformly distributed on RPE cells (G). Human POS bound to human RPE cells (B), which was accompanied by the coclustering of TLR4 (C). When RET was examined, RET was observed between the surface of the POS and TLR4 (D). When fluorescent bovine POS were incubated with human RPE cells, no coclustering with TLR4 could be detected. Thus, in contrast to the results found in Fig. 4 for CD36, only human POS promote the coclustering of TLR4 ($n = 9$) ($\times 736$).

anti-TLR4 mAb (clone HTA1216) at 37°C. Human POS were FITC-labeled as previously described (McLaren et al., 1993). FITC-conjugated POS were incubated for 20 min with RPE cells that were previously cultured on sterile microscope coverslips, gently washed, and then observed microscopically at 37°C. The concentration of POS was adjusted to give significant binding without obscuring other cellular details, which corresponded to a POS/cell ratio of 10:1. Fig. 2 B shows a low magnification micrograph illustrating the punctate POS labeling of an RPE monolayer. The fluorescent anti-TLR4 mAb was found to cluster near sites of POS binding (Fig. 2

C). To further establish this observation, RET microscopy was performed. As illustrated in Fig. 2 D, RET was observed between the FITC-labeled POS and TRITC-labeled anti-TLR4 reagent, indicating that these molecules are in close physical proximity.

To ascertain if functional TLR4 is important in TLR4 clustering on RPE surfaces, we employed a saturating dose of an inhibitory anti-TLR4 mAb (clone HTA125; Akashi et al., 2000; Tabeta et al., 2000). Cells were first labeled with a subsaturating dose (80 ng/ml) of rhodamine-conjugated anti-TLR4 mAb (clone HTA1216), as described above. This was followed by incubation

TABLE I
Quantitative Comparison of RET Intensities Using Various Experimental Conditions

Donor	Acceptor	Nature of RET Measurement (counts/s)		
POS type	TLR4	Individual POS	Single Cell	Monolayer Region
None	+	n/a	$0.1 \pm 0.1 \times 10^5$	$0.15 \pm 0.15 \times 10^5$
Human	–	$0.3 \pm 0.2 \times 10^4$	$0.11 \pm 0.08 \times 10^5$	$0.25 \pm 0.1 \times 10^5$
Human	+	$9.2 \pm 1.7 \times 10^4$	$1.6 \pm 2.4 \times 10^5$	$1.9 \pm 0.5 \times 10^5$
Bovine	+	$0.4 \pm 0.3 \times 10^4$	$0.13 \pm 0.1 \times 10^5$	$0.22 \pm 0.15 \times 10^5$
Background		$3.2 \pm 0.6 \times 10^3$	$0.8 \pm 0.1 \times 10^4$	$1.2 \pm 0.2 \times 10^4$

The value of n is 3–5 for these experiments. Columns three through five show quantitative measurements of RET intensities made on individual POS in a region of interest, a single RPE cell from a subconfluent monolayer, and a defined region of a confluent monolayer approximating one cell in size. For each experiment, 20–50 cells were measured. The background count rate was measured on an irrelevant part of a labeled slide. The background count rate was subtracted from the measurements shown. The human POS RET levels were significantly greater than those elicited by bovine POS using all three measurement methods ($P < 0.01$).

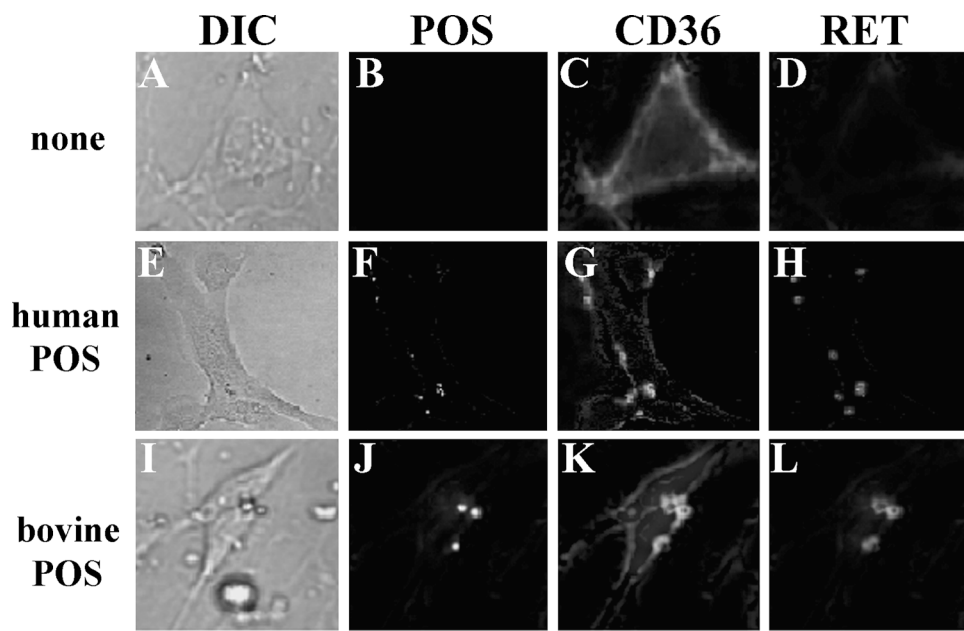


FIGURE 4. Human and bovine POS promote CD36 clustering on human RPE cells. Representative DIC (column 1), fluorescence (columns 2 and 3), and RET emission (column 4) micrographs are shown. RPE cells were labeled with TRITC-conjugated anti-CD36. Samples were then incubated in the absence of POS (A–D), FITC-conjugated human POS (E–H), or FITC-conjugated bovine POS (I–L). In the absence of POS, CD36 was distributed about the perimeter of RPE cells (C). Human POS bound to human RPE cells (F), which was accompanied by the coclustering of CD36 (G). When RET was evaluated, RET was observed between the surface of the POS and CD36 (H). When fluorescent bovine POS were incubated with human RPE cells, a similar set of observations were made (I–L). Thus, both human and bovine POS promote the coclustering of CD36 in their vicinity ($n = 8$) ($\times 736$).

with a saturating dose (30 $\mu\text{g}/\text{ml}$) of anti-TLR4 mAb clone HTA125 for 2 h at 37°C, followed by washing with medium. FITC-conjugated POS were added to cells and then observed microscopically at 37°C. Although POS bound to cells treated with a saturating dose of HTA125 (Fig. 2 F), TLR4 clustering at POS was not observed (Fig. 2 G) and RET between TLR4 and POS was not detected (Fig. 2 H). To control for potential nonspecific effects of mAb treatment, cells were labeled with anti-TLR4 mAb (clone HTA1216), as described above, followed by 30 $\mu\text{g}/\text{ml}$ of an isotype-matched control mAb. Under these conditions, TLR4 clustered as previously described in the absence of saturating anti-TLR4 treatment (unpublished data). Thus, saturating doses of an inhibitory antibody were found to block the clustering of TLR4 in response to POS binding.

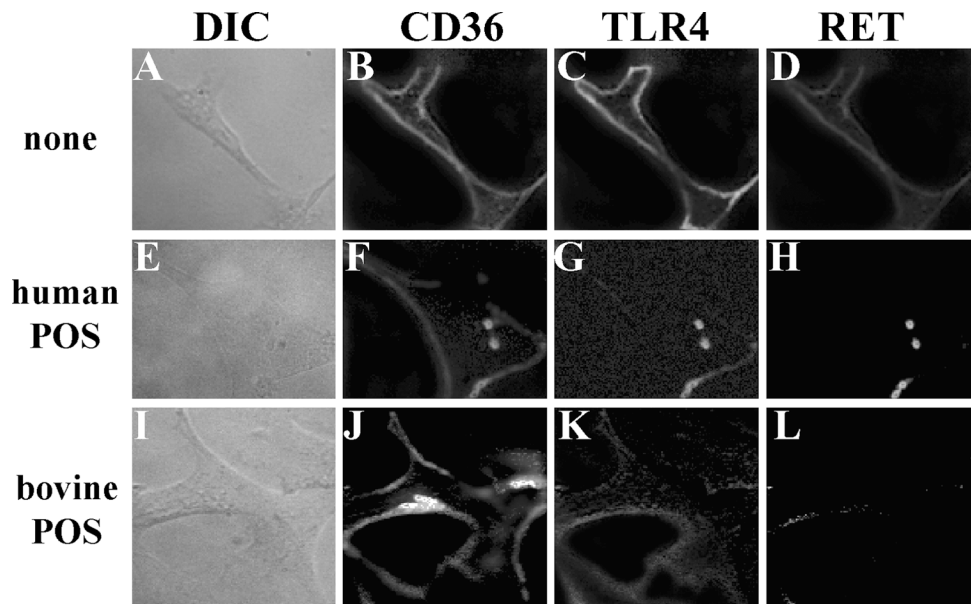
Subconfluent RPE cultures were also used as they were more readily labeled with mAb reagents and facilitated receptor analysis on individual cells. Fluorescently labeled POS were incubated with RPE cells as described in the preceding paragraph. Cells were labeled with TRITC-conjugated anti-TLR4 mAb. Fig. 3 G shows that TLR4 is uniformly distributed on untreated RPE cells. When human POS bind to RPE cells, TLR4 clusters at the site of POS binding (Fig. 3, A–C). However, when bovine POS bind to human RPE cells, TLR4 is unaffected (Fig. 3, I–L). Moreover, human POS, but not bovine POS, induce physical proximity between

TLR4 and POS (Fig. 3, D vs. L). Quantitative RET data are shown in Table I for these measurements as well as controls that omitted separately each fluorescent label. Table I also shows a parallel series of RET experiments performed on confluent cell monolayers. Thus, POS and TLR4 cocluster at the surface of sub-confluent RPE cells. Therefore, human, but not bovine, POS induce clustering of TLR4 at their binding sites. This suggests an important difference between the homologous and heterologous cell systems.

Clustering of TLR4 and CD36 at Sites of Outer Segment Binding: Differential Effects of Human and Bovine POS

Previous studies have reported that CD36 participates in the clearance of POS by RPE cells (Finnemann and Silverstein, 2001). In other systems, different membrane receptors collect at the site of phagocytosis (e.g., Petty et al., 1989). We therefore examined the cell surface distribution of CD36 on RPE cells in response to POS binding. On untreated cells, CD36 was predominantly found to be randomly distributed on RPE membranes (Fig. 4 C). Both human and bovine POS bound to human RPE cells, as confirmed microscopically (Fig. 4, F and J). Cells were also labeled with TRITC-conjugated anti-CD36. Sites of POS binding corresponded to sites of CD36 accumulation (Fig. 4, G and K), although some of the CD36 fluorescence remained uniformly distributed. Furthermore, the presence of RET between the POS and CD36 molecules suggests that the POS are

FIGURE 5. Human POS promote coclustering of CD36 and TLR4 on human RPE cells. Representative DIC (column 1), fluorescence (columns 2 and 3), and RET (column 4) micrographs are shown. RPE cells were labeled with TRITC-conjugated anti-CD36 and FITC-conjugated anti-TLR4. Samples were then incubated with human or bovine POS. CD36 and TLR4 were distributed about the perimeter of RPE cells (B and C). In the presence of human POS, CD36 (F) and TLR4 (G) coclustered on human RPE cells. RET microscopy demonstrated low levels of energy transfer on untreated cells (D). However, bright areas of RET could be observed on RPE cells in the presence of human POS (H). In contrast, bovine POS did not induce coclustering of CD36 and TLR4 or RET between these labels (J–L). Thus, human POS promote the coclustering of CD36 and TLR4 ($n = 6$) ($\times 736$).



in close physical proximity with CD36 (Fig. 4, H and L). Using quantitative microfluorometry, POS-to-CD36 RET intensities for whole cells of $1.6 \pm 0.4 \times 10^5$ and $1.7 \pm 0.5 \times 10^5$ counts per second were obtained for human and bovine POS, respectively, in comparison to controls omitting POS ($0.1 \pm 0.1 \times 10^5$ counts per second). Therefore, human and bovine POS induce clustering of CD36 at their binding sites, thus supporting the potential role of CD36 in handling POS.

A parallel series of experiments were performed using FITC-labeled anti-CD36 and TRITC-labeled anti-TLR4. RET microscopy indicated that CD36 and TLR4 were in close physical proximity on RPE cells at sites of POS binding (Fig. 5 H), as might be expected. However, RET was also observed on untreated RPE cells, indicating the molecular proximity of these molecules (Fig. 5 D). These RET findings for CD36-to-TLR4 proximity in the absence and presence of POS were confirmed using quantitative microfluorometry of separate whole cells ($1.8 \pm 0.5 \times 10^5$ vs. $1.9 \pm 0.3 \times 10^5$ counts per second, respectively). Thus, the level of RET is indistinguishable in the presence and absence of receptor clustering due to POS. This observation suggests that TLR4 and CD36 may be components of the same supramolecular complex on RPE cell membranes. The substantial reduction in RET intensity on cells incubated with bovine POS is likely due to the redistribution of CD36 to the POS and the lack of TLR4 accumulation at these sites.

Arrival of TLR4 at Sites of Human POS Binding Coincides with Metabolic and Calcium Signaling Events

To examine the potential role of RPE TLR4 in POS signaling events, we performed simultaneous kinetic studies of receptor accumulation at POS binding sites and intracellular metabolic and calcium signaling processes. Cells were labeled with FITC-anti-CD36, TRITC-anti-TLR4, and/or the calcium-sensitive probe indo-IAM. The fluorescence intensities of the CD36, TLR4, or the RET between these two labels was measured within a circular region including and surrounding individual bound POS, which was selected using region-of-interest software. Kinetic changes in the fluorescence intensity in the region-of-interest were recorded immediately after POS addition to the RPE cells. Although the total fluorescence intensity of the cell does not change (unpublished data), the fluorescence intensity surrounding the POS does change. Fig. 6 A shows representative kinetic studies of CD36 and TLR4 accumulation at a POS. The curves appear as dashed lines because the filter and dichroic mirror set was moved between the two positions to detect these two fluorochromes during data acquisition. Fig. 6 A shows that CD36 arrives at the sites of POS binding before TLR4, with an approximate lag time of 2–2.5 min. The RET signal between the CD36 and TLR4 labels initially falls (not depicted); this is followed by a subsequent increase in RET in the vicinity of the POS (Fig. 6, B and C), as anticipated by Fig. 5 H. When RET emission and NAD(P)H autofluo-

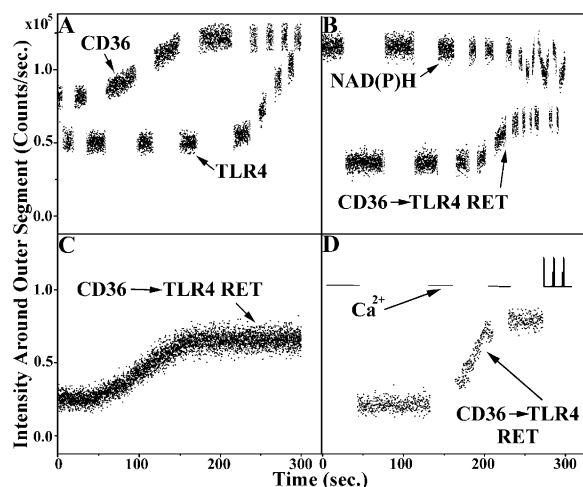


FIGURE 6. Quantitative kinetic analysis of receptor complex assembly and signaling at sites of POS binding. Using a PMT detector, photon count rates in the vicinity of bound POS were measured. Intensity is plotted at the ordinate, whereas time is given at the abscissa. The region surrounding the POS was selected by an iris in a back focal plane of the microscope. (A) Samples were labeled with both TRITC-conjugated anti-CD36 and FITC-conjugated anti-TLR4. The kinetics of CD36 and TLR4 accumulation at the site of POS binding is shown. CD36 arrives at the site of POS binding before TLR4. (B) Correspondence between the times of anti-CD36-to-anti-TLR4 RET and the exhibition of NAD(P)H oscillations during POS binding. (C) Kinetics of RET acquisition between anti-CD36 and anti-TLR4 at a site of POS binding. (D) Cells were labeled with anti-CD36 and anti-TLR4 and with the calcium indicator indo-1. Correspondence between the times of anti-CD36-to-anti-TLR4 RET and the initiation of calcium spikes in RPE cells ($n > 4$).

rescence were simultaneously recorded, high frequency NAD(P)H oscillations were detected after CD36 and TLR4 came into close proximity on RPE cells (Fig. 6 B). Similarly, calcium spikes, as judged by the increased emission of indo-1, are observed in RPE cells, but only

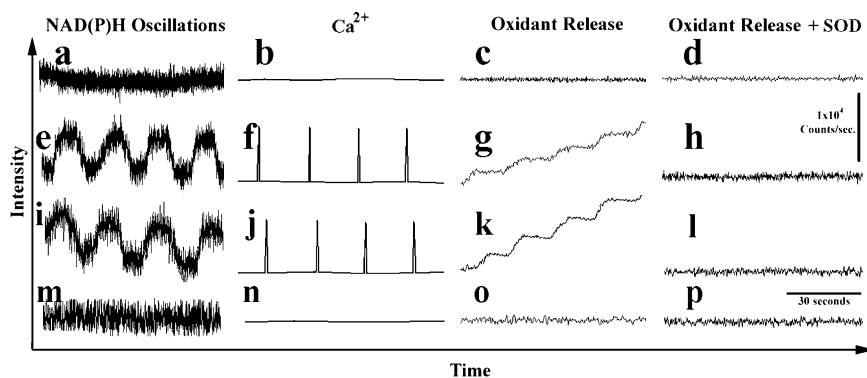


FIGURE 7. Representative NAD(P)H oscillations and oxidant release profiles of RPE cells. Human RPE cells were incubated with bovine (a–d) or human (e–p) POS. Cells were monitored for NAD(P)H concentration (a, e, i, and m), intracellular calcium levels (b, f, j, and n), oxidant release (c, g, k, and o), and oxidant release in the presence of superoxide dismutase (SOD) (d, h, l, and p). Although bovine POS did not affect NAD(P)H and calcium signals of human RPE cells (a and b), these signals were apparent when cells were incubated with human POS (e and f). The release of oxidants was detected with H₂-

TMRose as previously described (Kindzelskii et al., 1998). In parallel with their effects on signaling, bovine POS did not induce oxidant release (c), whereas human POS promoted oxidant release (g). This oxidant release could be inhibited by SOD (h). The ability of POS to stimulate metabolic oscillations, calcium signaling, and ROM release was not affected by prior labeling with anti-TLR4 (clone HTA1216; 80 ng/ml) (i–l). However, labeling with anti-TLR4 clone HTA1216 followed with treatment with anti-TLR4 clone HTA125 at 30 μ g/ml blocked metabolic oscillations, calcium signaling, and ROM release (m–o) ($n = 5$). Bar, 30 s.

after the formation of proximity complexes of CD36 and TLR4 (Fig. 6 D) at POS binding sites. Thus, TLR4 appears to be one component necessary for the initiation of metabolic and calcium signaling in response to human POS binding.

Apparent Role of TLR4 Clustering in RPE Signaling in Response to POS

If TLR4 clustering is required for certain elements of POS signaling within RPE cells, then bovine POS should not be capable of eliciting these changes. To test this hypothesis, bovine POS were incubated with human RPE cells. Bovine POS were unable to induce NAD(P)H oscillations or calcium spikes in RPE cells (Fig. 7, a and b). RPE cells were also incubated with human POS. In this case, however, both NAD(P)H oscillations and calcium spikes were observed (Fig. 7, e and f). Furthermore, NAD(P)H oscillations and calcium spikes were observed on confluent human RPE monolayers in regions with bound human POS (Fig. 8). The metabolic oscillations and calcium signals in cell monolayers were substantially greater in amplitude than those of single cells, which may be due to the fact that multiple cells (approximately three to five) are illuminated during these observations on monolayers. These findings are consistent with the proposed role of TLR4 in these two elements of RPE cell signaling in response to human POS.

The experiments shown in Fig. 2 demonstrate that an inhibitory anti-TLR4 mAb blocks TLR4 clustering in response to human POS. Therefore, we tested the ability of this reagent to effect metabolic and calcium signaling. When cells were treated with anti-TLR4 clone HTA1216 at 80 ng/ml, no effect on metabolic oscillations and calcium signaling in response to human POS was noted (Fig. 7, i and j). However, when RPE cells were exposed to anti-TLR4 clone HTA1216 at 80 ng/

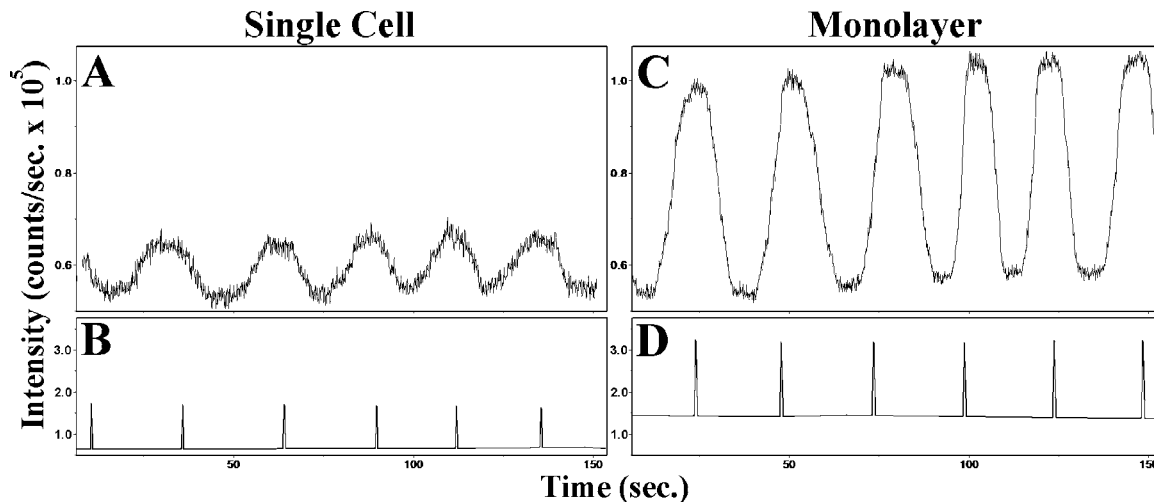


FIGURE 8. Representative NAD(P)H oscillations and calcium spikes of RPE cells. The intensity is plotted at the ordinate, whereas the time in seconds is given at the abscissa. Experiments were performed as described in the preceding figure. Human RPE cells were incubated with human POS followed by analyses of NAD(P)H oscillations (A and C) or calcium spikes (B and D). Single cells (A and B) and cell layers (C and D) were evaluated. In the case of cell layers, more than one cell was illuminated, thereby leading to the higher amplitudes of the oscillations. Nonetheless, similar behaviors were noted for single cells and monolayers.

ml, washed, and then incubated with 30 $\mu\text{g}/\text{ml}$ anti-TLR4 clone HTA125 for 20 min, metabolic oscillations and calcium signaling in response to human POS were inhibited (Fig. 7, m and n). Thus, several lines of evidence support the idea that TLR4 accumulation at sites of POS binding participate in RPE responses for the homologous human POS/human RPE cell system.

Human, but Not Bovine, POS Promote the Production of ROM by Human RPE Cells, Which Can Be Blocked by an Inhibitory Anti-TLR4 mAb

Inasmuch as TLRs participate in host defense by leukocytes (Akira, 2000; Schuster and Nelson, 2000; Dobrovolskaia and Vogel, 2002), RPE cells have been reported to produce superoxide anions (Dorey et al., 1989; Miceli et al., 1994; Tate et al., 1995; Wu and Rao, 1999), and metabolic oscillations and calcium signaling have been correlated with ROM production (Petty, 2001), we tested the hypothesis that POS promote ROM release. ROM production by RPE cells was measured as previously described (Kindzelskii et al., 1998). Human RPE cells that were untreated (not depicted) or treated with bovine POS (Fig. 7 c) did not produce ROM that could be detected by this assay. However, human POS did stimulate ROM production by human RPE cells (Fig. 7 g), which could be inhibited by the ROM scavenger superoxide dismutase (SOD) (Fig. 7 h). To reconfirm the functional role of TLR4 in this response to human POS, cells were treated with anti-TLR4 mAbs. When cells were treated with anti-TLR4 clone HTA1216, ROM production in response to human POS was noted, as in the absence of this reagent. However, when RPE cells were exposed to anti-TLR4

clone HTA1216 at 80 ng/ml, washed, and then incubated with 30 $\mu\text{g}/\text{ml}$ anti-TLR4 clone HTA125 for 20 min, ROM production in response to human POS was blocked (Fig. 7 o), which could not be distinguished from negative controls (Fig. 7 p). Thus, this physiological response to human POS is apparently tied to TLR4 function and transmembrane signaling, which is likely important in the management of POS by RPE cells.

DISCUSSION

Although human TLRs were discovered only a few years ago, they have come to occupy center stage in innate immunity against invading pathogens (Akira, 2000; Schuster and Nelson, 2000; Dobrovolskaia and Vogel, 2002). The breadth of the importance of TLR genes in humans is not yet known, but given the heterogeneity of *Toll* functions found in *Drosophila* as well as the broad functions of other pattern recognition receptors, the TLR proteins are likely to occupy several key biological roles. Specifically, human TLR4 participates in cellular responses to the exogenous substances LPS of Gram-negative bacteria, lipoteichoic acid of Gram-positive bacteria, and the F protein of respiratory syncytial virus and as a receptor for the endogenous substances HSP60 (and certain homologous proteins), the fibronectin extra domain A, and hyaluronan (Barton and Medzhitov, 2002; Beg, 2002; Johnson et al., 2003). In a preliminary report, we previously noted that TLR4 is present on RPE cells (Petty et al., 2003). The present study extends the repertoire of TLRs to potentially include vision-related processes of RPE cells. Our work suggests that TLR4 of human RPE cells participates in the cellular handling of human POS on both confluent

cultures, which resemble normal physiological conditions, and subconfluent cultures, which resemble the migratory RPE cells associated with certain disease processes (Hogg et al., 2002). Several lines of experimental evidence now support a possible role of TLR4 in RPE responses to POS. These include (a) TLR4 clusters on human RPE surfaces in response to human POS, but not bovine POS, (b) TLR4 arrives at human POS just before signal transduction, and (c) an inhibitory anti-TLR4 mAb blocks TLR4 clustering, calcium signaling, and metabolic responses of RPE cells to human POS.

Several steps are involved in the RPE-mediated clearance of POS. These include binding to the RPE surface, phagocytosis, and lysosomal destruction. Although binding of POS to RPE cells occurs rapidly (<1 h), phagocytosis requires roughly 3 h (Hall and Abrams, 1987). As all of the above observations were made less than 1 h. after POS addition, they are relevant to the early events during POS-to-RPE cell interactions. One fundamental requirement of transmembrane signaling is that the receptor is engaged before or during the production of a transmembrane signal. Our experiments show that calcium and metabolic changes are preceded by CD36 and TLR4 accumulation at sites of POS binding. These findings are consistent with a possible role of these molecules in signaling in response to POS.

Several RPE membrane proteins may participate in the clearance of POS. The membrane protein MERTK plays a central role in generating signals leading to POS phagocytosis (Feng et al., 2002). The role of MERTK in eye disease has been further established by genetic studies (Gal et al., 2000; D'Cruz et al., 2000). Evidence has suggested that the membrane proteins CD36 and $\alpha_v\beta_5$ participate in POS binding to RPE cells (Finnemann et al., 1997; Finnemann and Silverstein, 2001). The role of CD36 in recognition is not surprising since this is a pattern recognition receptor of broad specificity (Febbraio et al., 2001). However, the fact that RET was detected between CD36 and TLR4 was surprising; this suggests that they may cluster in the same cell membrane domain or are physically associated with one another. In either case, it will be interesting to determine if CD36 and TLR4 have cooperative roles in other biological systems. Our data suggest that TLR4 may participate in the clearance of POS, such as the stimulation of additional RPE signaling pathways necessary for the degradation of POS. Therefore, we speculate that POS clearance is managed by a supramolecular complex of membrane proteins. This may be analogous to the handling of LPS by leukocytes, which involves multiple membrane-associated proteins, including CD14, TLR4, MD-2, and β -2 integrins; several of these proteins are already known to interact with one another in cell membranes (Petty et al., 2002). In

this system, CD14, TLR4, and β -2 integrins are thought to be primarily, but not exclusively, responsible for LPS binding, signaling for cytokine up-regulation, and phagocytic uptake, respectively. Similarly, the POS clearance may involve CD36 as a participatory recognition molecule, MERTK as a phagocytosis signaling molecule, and TLR4 as an activating stimulus, which is specific for a molecular pattern on human POS. Although integrin $\alpha_v\beta_5$ may not participate directly in phagocytosis (Hall et al., 2003), it may participate in other aspects of POS management, such as facilitating and integrating signal transduction, as in the leukocyte system (Vogel et al., 2001).

Although we suggest that POS are managed by a supramolecular assembly of membrane components, this complex may not be static. For example, our studies have also shown that CD36 arrives at POS before TLR4. This finding suggests that CD36 plays an earlier role managing POS whereas TLR4 appears to play a downstream role including calcium and metabolic signaling and ROM production. The apparent role of TLR4 in calcium and metabolic signaling is also supported by the fact that an inhibitory anti-TLR4 mAb blocks these signals. Thus, RPE cell TLR4 molecules appear to be an important contributor to certain aspects in the management of POS. Further analysis of proteins that may contribute to POS clearance mechanisms, including their supramolecular structures and their dynamic regulation, such as the timing of MERTK recruitment, as well as their subsequent disassembly and recycling will be future challenges in understanding this biological pathway.

Recent studies have indicated that TLR4 of leukocytes stimulates the cell functions, such as the production of ROM (Hayashi et al., 2003; Werling et al., 2004). Furthermore, analysis of mice genetically deficient in TLR4 indicates that TLR4 contributes to the induction of ROM production in leukocytes (Remer et al., 2003). Previous studies have indicated that RPE cells are capable of generating ROM under certain conditions (Dorey et al., 1989; Miceli et al., 1994; Tate et al., 1995; Wu and Rao, 1999). Therefore, it seems possible that TLR4 clustering and transmembrane signaling contribute to ROM production. Our studies have shown that RPE cells produce ROM in response to POS. Furthermore, an inhibitory anti-TLR4 mAb blocks ROM production in response to POS. Our studies suggest that in RPE cells, as in leukocytes, TLR4 can participate in transmembrane signaling leading to ROM production.

Although RPE cells can produce ROM (Dorey et al., 1989; Miceli et al., 1994; Tate et al., 1995; Wu and Rao, 1999), this process has not been studied in detail in the RPE system. In the present study, we have observed significant rates of ROM generation, but only with the homologous human RPE-human POS system. Although a

previous study noted ROM production in the heterologous system (Dorey et al., 1989), the rate of ROM production was much lower than our observations in the homologous system. Although our single cell assay did not detect ROM production over several minutes for the heterologous system, a previous study (Dorey et al., 1989) of millions of cells detected ROM production over a period of hours. Thus, our findings are consistent with prior reports and indicate that the homologous system provides more robust results. Furthermore, a significant level of oxidant production is only observed using human POS, which is accompanied by TLR4 recruitment, calcium signaling, and metabolic changes. As bovine POS do not elicit these responses, it would appear that the heterologous bovine POS–human RPE system is not a good model for certain aspects of POS clearance. Thus, results with heterologous systems should be confirmed using more physiological homologous systems. In the present study, the bovine POS–human RPE system served as a negative control. We believe that prior studies using heterologous systems necessarily missed the involvement of TLRs in POS handling and downstream RPE functions such as ROM production. Human POS have the ability to induce metabolic changes, calcium signals, and ROM production in human RPE cells, which are characteristics of immunologically primed neutrophils (Petty, 2001). We speculate that TLR4 clustering may also play important roles in proinflammatory pathophysiologic changes in the eye that accompany retinal diseases such as uveitis, age-related macular degeneration, and proliferative vitreoretinopathy. From a more general perspective, our studies on TLR4, transmembrane signaling, and oxidant release lead to the speculation that POS binding creates a local proinflammatory environment in the eye, which might be triggered to form a “full-blown,” damaging inflammatory response by small perturbations (cytokines, LPS, etc.) in the local environment.

Recent animal experiments are consistent with the potential role of TLR4 and proinflammatory conditions as modulators of eye disease. The *mnd* (motor neuron degeneration) mouse, which displays retinal disease at 1 mo of age accompanied by more general neurologic aberrations at 6 mo (Messer et al., 1995), is a good model of neuronal ceroid lipofuscinosis (Batten disease) (Chang et al., 1994). It has been shown that when C57BL/6.KB2-*mnd* mice are out-crossed with AKR/J mice (TLR4 positive), the disease becomes more aggressive, whereas out-crossing with the C3H/HeJ background (TLR4 defective) has no effect on the timing of disease (Messer et al., 1999; Bihl et al., 2001). Recently, C57BL/6.KB2-*mnd* mice have been shown to possess a novel mutation in TLR4 leading to hyporesponsiveness to LPS (Bihl et al., 2001). Thus, the lack

of functional TLR4 appears to have a protective influence in these animals. The TLR4-mediated signals noted above may contribute to the accelerated retinal degeneration of TLR4-positive animals. Thus, the proinflammatory conditions found during POS turnover may constitute a previously unsuspected modulator of disease aggressiveness acting through the TLR4 pathway outlined above. Recent studies have associated TLR4 polymorphisms with the risk of atherogenesis (Kiechl et al., 2003), which may have an important inflammatory component. These findings in animals and humans and our present in vitro studies raise the possibility that TLR4 polymorphisms in humans may contribute to the severity of eye disease, especially ROM-related eye diseases such as age-related macular degeneration.

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