Replication of *Mycobacterium Tuberculosis* in Retinal Pigment Epithelium

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Tuberculosis (TB) remains a global health problem, with almost one-third of the world’s population being infected. The disease presents primarily as pulmonary TB and, in approximately 20% of patients, as extrapulmonary disease. After *Mycobacterium tuberculosis* has been inhaled, the organisms undergo phagocytosis by alveolar macrophages, resulting in bacterial containment. In some patients, the bacilli may disseminate systemically, establishing latent infection at extrapulmonary sites, with the potential to reactivate at a later time. Reactivation of latent TB usually occurs when the host’s immune system is compromised.

**RESULTS** At day 0, an equal number of intracellular *M tuberculosis* was observed per THP-1 and RPE cells (0.45 and 0.35 *M tuberculosis* per RPE and THP-1 cells, respectively). Mean (SD) number of intracellular *M tuberculosis* at day 5 was 1.9 (0.03) and 3.3 (0.01) per RPE and THP-1 cells, respectively (*P* < .001). Viability of infected RPE was significantly greater than that of THP-1 cells at day 5 (viable cells: 17% THP-1 vs 73% [4%] RPE, *P* < .05). Expression of TLR2 and TLR4 was detected in both cell types after 12 hours of exposure. Inhibition of TLR2 and TLR4 reduced intracellular *M tuberculosis* counts in RPE but not in THP-1 cells.

**CONCLUSIONS AND RELEVANCE** *Mycobacterium tuberculosis* is phagocytized by RPE to a similar extent as in macrophages. However, RPE cells are better able to control bacillary growth and RPE cell survival is greater than that of THP-1 cells following mycobacterial infection, suggesting that RPE can serve as a reservoir for intraocular *M tuberculosis* infection.
Recognition and phagocytosis of mycobacteria by macrophages is mediated in part by cellular pattern recognition receptors, toll-like receptors (TLRs), and others, such as complement receptors, macrophage mannose receptors, and adenosine receptors.8,7,8 Among these, TLRs are critical in M tuberculosis recognition and in initiating innate and adaptive immune responses.9-13 Retinal pigment epithelium cells possess robust phagocytic functions and share several surface molecules required for bacterial phagocytosis with macrophages, including TLRs.12,13 Whether RPE may use such molecules in phagocytosis of bacilli that reach the intraocular tissues during blood-borne dissemination of the organism from the lung remains to be determined.6 Mycobacterium tuberculosis H37 has been used for decades to study the pathogenesis of TB. Serial passage of M tuberculosis H37 through media with different pH levels allows its dissociation into 2 forms: the virulent M tuberculosis H37Rv and the attenuated M tuberculosis H37Ra.14 The H37Ra form has been used extensively to study the mechanisms of M tuberculosis phagocytosis by resident reticuloendothelial cells.15 Multiple studies16,17 have shown that macrophages use identical surface receptors for phagocytosis of both virulent (H37Rv) and avirulent (H37Ra) strains.

In the present study, we used M tuberculosis H37Ra to investigate the bacillary phagocytosis, replication, and cytotoxic effects in RPE cells compared with macrophages. We also studied the role of TLR2 and TLR4 in the phagocytosis of M tuberculosis by RPE.

Methods
All experiments and procedures were conducted in adherence to the Declaration of Helsinki. The institutional review board of the University of Southern California reviewed and accepted the protocol to use human tissue materials.

RPE and Monocyte/Macrophage Cell Cultures
Human fetal RPE cells were extracted from 20-week fetus eyes (Advanced Bioscience Resources Inc) according to established protocols.18 Passage 2 cells were transferred to 75-mL flasks and grown to confluence in Dulbecco Modified Eagle medium (DMEM) (Lonza BioWhittaker) containing 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies), 1-glutamine (1mM), hydroxethyl piperaazineethanesulfonic acid buffer (10mM), and penicillin G sodium and streptomycin sulfate (50μg/mL). After attaining confluence, RPE cells were passaged 2 additional times in antibiotic-free DMEM containing 10% heat-inactivated fetal bovine serum. The cells were then transferred at 2 × 10⁵ cells/well, either into 4-chamber slides (BD Biosciences) for acid-fast and immunofluorescence staining or into 12-well plates (BD Biosciences) for intracellular M tuberculosis load analysis. The medium was changed twice each week during all experiments unless specified otherwise. Before all medium changes, cells were washed 3 times with 37°C culture medium to remove nonadherent cells. Acute monocytic leukemia cell line (THP-1) (ATCC) was cultured in antibiotic-free Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) containing 10% heat-inactivated fetal bovine serum. The medium was changed twice each week. During the medium change, the cell cultures were evaluated by phase-contrast microscopy using trypan blue, and the number of nonviable cells was less than 5% in all experiments before M tuberculosis was added to the cultures. Adhesion of THP-1 to culture plates was attained by adding 12 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Corp) to each well. The slide or the plates were washed with phosphate-buffered saline (37°C) twice before seeding. The THP-1 cells were plated at 5 × 10⁵ cells/well in 4-chamber slides or 12-well plates; they were left still for 60 minutes with PMA-containing medium and then washed with 37°C phosphate-buffered saline to remove nonadherent cells. The THP-1 cells were evaluated daily for morphologic changes, including cytoplasmic projections.

M tuberculosis H37Ra Infection
The H37Ra (ATCC) was cultured in Middlebrook 7H9 broth (BD Biosciences) to midlog phase (optical density approximately 0.5) and stored in liquid nitrogen. After thawing, mycobacteria were cultured in Middlebrook 7H9 broth for 1 to 2 days and sonicated for 2 minutes before infecting RPE and THP-1 cells. Mycobacterial culture density was quantified by spectrophotometry, and both RPE and THP-1 cells were infected with a multiplicity of infection (MOI) of 5:1. After a 12-hour exposure of cells to M tuberculosis, the supernatants were collected and the number of live and floating dead cells was enumerated by trypan blue exclusion. After supernatants were collected at 12 hours, the attached cells in the culture wells and chamber slides were washed with culture medium containing streptomycin, 200 μg/mL, to kill noninternalized mycobacteria. After removal of nonadherent mycobacteria, complete medium (DMEM for RPE and RPMI 1640 for THP-1) containing streptomycin (20 μg/mL) was added to the culture wells for 24 hours to kill the remaining extracellular mycobacteria attached to the cell surface. At this time and beyond (at 36 and 132 hours), the medium was replaced with an antibiotic-free medium.

Cell Viability Assessment and Enumeration of the Viable Intracellular M tuberculosis
Cell supernatants were collected and the numbers of the viable and nonviable cells were counted in triplicate at 12, 36, and 132 hours by phase-contrast microscopy using trypan blue exclusion. To study the number of live intracellular mycobacteria, attached THP-1 and RPE cells were lysed by adding 1 mL of 1% Triton X-100 (Sigma-Aldrich Corp) to each well for 5 minutes. Cell lysates were harvested and cultured on Middlebrook 7H10 agar (BD Biosciences) in serial dilutions of the original lysate. All cultures were performed in triplicate. Agar plates were cultivated in a 37°C humidified incubator with 5% carbon dioxide for 3 to 4 weeks before determination of colony-forming units (CFUs). Total CFU counts were also determined using the same method by lysing the cells from the overlying medium. Because the 12-hour supernatants contained both floating cells, possibly containing mycobacteria,
and the noninternalized mycobacteria, CFUs for the supernatant were not determined at this time.

**TLR2 and TLR4 Expression and Inhibition in Phagocytosis**

The RPE and THP-1 cells were cultured on slides in removable chambers for acid-fast staining and immunofluorescence staining to evaluate expression of TLR2 and TLR4. The RPE and THP-1 cells infected with *M tuberculosis* at an MOI of 5:1 were fixed with 4°C methanol for 10 minutes and stained with auramine-rhodamine (BD Biosciences) at 36 and 132 hours after infection. Immunohistochemical examination of the cells for TLR2 and TLR4 expression was performed after 6, 12, 36, and 132 hours of *M tuberculosis* exposure. At these time points, attached cells were washed 3 times with streptomycin-containing culture medium and fixed with 4°C methanol for 10 minutes. Fixed cells were stained immediately or kept overnight at 4°C before staining. Slides were blocked with phosphate-buffered saline containing 5% fetal bovine serum and 0.1% Triton X-100 for 30 minutes before overnight incubation with primary antibodies, including antihuman-TLR2 antibody and antihuman-TLR4 antibody (Abcam). Slides were then blocked with secondary antibody, counterstained with 4',6-diamidino-2-phenylindole, and examined within 24 hours by confocal fluorescent microscopy.

The role of TLR2 and TLR4 molecules in RPE and THP-1 phagocytosis of *M tuberculosis* was evaluated using 50 μg/mL of oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC) (TLR4 and TLR2 inhibitor; InvivoGen). The cells were preexposed to the OxPAPC for 6 hours before *M tuberculosis* was added to the medium. The TLR2/TLR4 blocker was washed before *M tuberculosis* exposure, as described above. The cells were exposed to *M tuberculosis* for 12 hours. The OxPAPC-pretreated THP-1 and RPE cells were harvested at 6, 12, and 36 hours after *M tuberculosis* infection for CFU determination, as described above.

**Statistical Analysis**

All experiments were performed in triplicate with appropriate positive and negative controls. An unpaired 2-tailed *t* test was used to compare the numeric data in 2 groups. The level of significance was considered to be *P* < .05.

**Results**

**Phagocytosis and Intracellular Replication**

The THP-1 cells are frequently used as an alveolar macrophage model for *M tuberculosis* infection. Differentiated THP-1 cells, as described below, share several key features of alveolar macrophages following an encounter with *M tuberculosis*. To compare the intracellular load of *M tuberculosis* in RPE and THP-1 cells, both cell cultures were infected with *M tuberculosis* at an MOI of 1:1 and 5:1 and lysed at various time points for CFU determination. At 12 hours after *M tuberculosis* infection, the CFU count per cell was 0.45 and 0.35 for RPE and the THP-1macrophages, respectively (*P* > .05), suggesting that the rate of *M tuberculosis* phagocytosis by RPE and THP-1 cells is similar. However, the intracellular bacillary load was higher in THP-1 cells compared with RPE cells at 36 and 132 hours after infection (Figure 1). Thus, at an MOI of 5:1, the mean (SD) CFUs per cell were 0.9 (0.04) and 2.2 (0.1) for RPE and THP-1 cells, respectively (*t* test, *P* = .003) at 36 hours. At 132 hours after infection (MOI 5:1), the CFU count was 1.9 (0.03) and 3.3 (0.01) in RPE and THP-1 cells, respectively (*P* < .001) (Figure 2).

**Cellular Viability**

Retinal pigment epithelium and THP-1 cell death rates were significantly different at all time points after exposure. At 12 hours, 39% and 66% of the THP-1 and RPE cells were alive, respectively (*P* < .05). Faster bacillary growth in THP-1 cells was associated with a higher number of dead cells compared with RPE (17% viable THP-1 cells vs 73% viable RPE cells at 132 hours at MOI 5:1 (Figure 3).)

**TLR2 and TLR4 Expression**

The expression of TLR2 and TLR4 on RPE and THP-1 cells was studied by immunofluorescent staining for these molecules at various time points after *M tuberculosis* exposure. Both TLR2 and TLR4 were expressed maximally at 12 hours after *M tuberculosis* exposure in RPE and THP-1 cells, and their expression decreased in each cell type at the 36-hour time point (Figure 4).
Replication of M tuberculosis in Retinal Pigment

The initial Mycobacterium tuberculosis to live RPE and M tuberculosis to live THP-1 ratio was equivalent, but the ratio of M tuberculosis per live cell increased more robustly in THP-1 macrophages compared with RPE at day 5 (132 hours) ($P < .001$). Colony-forming unit determination and cellular viability measurements were performed in triplicate cultures. Limit lines indicate SD.

Effect of TLR2 and TLR4 Inhibition

In the present study, TLR2 and TLR4 were inhibited chemically to study their role in the recognition and phagocytosis of M tuberculosis by RPE and THP-1 cells. Blockade of TLR2 and TLR4 by OxPAPC did not significantly alter the CFU count in THP-1 cells 36 hours after exposure ($P = .13$). However, at 36 hours after M tuberculosis infection of RPE, the number of internalized mycobacteria in TLR2- and TLR4-inhibited cells was 50% of that in negative control cultures ($P = .002$) (Figure 5). The results were confirmed by polymerase chain reaction quantification of the M tuberculosis genome copy number in THP-1 and RPE cells with and without treatment by TLR2- and TLR4-blocking agents. Mycobacterium tuberculosis genome copy number in RPE lysate of TLR2- and TLR4-inhibited cells was 60% of that in noninhibited cells 36 hours after M tuberculosis exposure.

Discussion

The present study reveals that M tuberculosis is readily phagocytized by RPE cells at a rate similar to that of macrophages and that M tuberculosis does not significantly alter RPE viability following infection. These data suggest that RPE can harbor M tuberculosis following phagocytosis of bacilli and that phagocytosis is dependent on expression of TLR2 and/or TLR4 by the RPE. In a histopathologic study of an enucleated globe with panuveitis, Rao et al$^{16}$ used quantitative polymerase chain reaction to detect the presence of the M tuberculosis genome within RPE cells. The authors suggested that M tuberculosis is preferentially localized in RPE, perpetuating intraocular inflammation from these cells. Retinal pigment epithelium is a phagocytic cell similar to macrophages. Resident choroidal reticuloendothelial cells can also be natural phagocytic cells for M tuberculosis during ocular dissemination, and choroidal tubercles are more likely caused by reactivation of M tuberculosis from this source. However, the clinical pattern of TB-associated multifocal serpiginoid choroiditis supports RPE localization of M tuberculosis.20 The results of the present study suggest that RPE can act as an M tuberculosis reservoir in the eye and provide a niche for delayed reactivation and multiplication of the pathogen; however, the mechanism for such reactivation is not clear.

Our results indicate that RPE cells possess the receptors for binding to M tuberculosis as well as the machinery to phagocytize the organism (Figures 4 and 5). A diverse family of phagocytic pattern recognition receptors, including mannose receptors and cytosolic TLRs, facilitate M tuberculosis recognition and phagocytosis by macrophages and dendritic cells,21 Retinal pigment epithelium cells express several of these receptors.22,23 Considering the comparable number of intracellular M tuberculosis in RPE and macrophages in the current study, it is possible that the receptors in both cell types have similar efficiency for detection and phagocytosis of M tuberculosis. These results suggest that strategies can be sought to thwart M tuberculosis phagocytosis by RPE and to prevent intraocular TB.24-27

In the present study, fewer intracellular bacilli were detected in RPE compared with macrophages 5 days after initial exposure to M tuberculosis (Figure 2). In addition, RPE viability was greater compared with that of macrophages (Figure 3). It is likely that macrophages allow more M tuberculosis growth, ultimately leading to the death of the cells.28 Mycobacterium tuberculosis can reduce its metabolic activity to evade intracellular detection and triggering of cell death pathways.29,30
Our results indicate that adaptation of *M tuberculosis* H37Ra to the intra-RPE environment and escape from death pathways could be more efficient than that in the intramacrophage environment. This phenomenon confirms clinical observations of normal-appearing RPE in patients who later present with *M tuberculosis* chorioretinitis. Further characterization of bacillary persistence and cellular death mechanisms in *M tuberculosis*-exposed RPE may eventually help in treating intraocular TB.

Toll-like receptors are key receptor families expressed by alveolar macrophages, and the receptors form an initial line of defense against invading pathogens. In addition, TLR2 and TLR4 are capable of recognizing pathogen-associated molecular patterns expressed by *M tuberculosis* and in initiating innate immune response to the mycobacteria. In pursuing the mechanisms of RPE infection by mycobacteria and because of the importance of TLR2 and TLR4 in regulation of macrophage response to *M tuberculosis*, we chose to identify the role of TLR2 and TLR4 in the interaction between RPE and *M tuberculosis*. In the present study, the human RPE cultures showed expression of TLR2 and TLR4 after 12 hours of exposure to *M. tuberculosis*. In addition, a lower number of intra-RPE *M tuberculosis* was observed after TLR2 and TLR4 blockade. These data suggest that these molecules are pivotal in *M tuberculosis* recognition and internalization by RPE cells. However, additional studies are needed to further examine the role of each TLR in *M tuberculosis* phagocytosis.

Because of the essentially equivalent behavior of H37Ra and H37Rv in early recognition by macrophages, we chose to use H37Ra to investigate the phagocytosis of *M tuberculosis* by RPE and to characterize the role of TLRs in this process. We believe that these studies provide satisfactory proof of principle that RPE is capable of phagocytosis of tubercle bacilli. However, our results should be viewed carefully because of the above-mentioned inherent differences between virulent and avirulent *M tuberculosis* in initiation of immune recognition pathways. Studies to better characterize the local immune response to intra-RPE TB should be carried out with H37Rv.
Conclusions

The TLR2 and TLR4 expression-dependent phagocytosis of M. tuberculosis by RPE and survival of the RPE suggest that M. tuberculosis can be harbored in RPE and that these cells could be the likely site for latent M tuberculosis as well as the site for subsequent reactivation and development of posterior uveitis. However, further clarification of the mechanisms of the latent TB infection in RPE cells is needed to develop effective treatments for intracellular tuberculosis.