Localization of TLR2 and MyD88 to *Chlamydia trachomatis* Inclusions

**EVIDENCE FOR SIGNALING BY INTRACELLULAR TLR2 DURING INFECTION WITH AN OBLIGATE INTRACELLULAR PATHOGEN**

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*Chlamydia trachomatis* is an obligate intracellular Gram-negative pathogen and the etiologic agent of significant ocular and genital tract diseases. Chlamydiae primarily infect epithelial cells, and the inflammatory response of these cells to the infection directs both the innate and adaptive immune response. This study focused on determining the cellular immune receptors involved in the early events following infection with the L2 serovar of *C. trachomatis*. We found that dominant negative MyD88 inhibited interleukin-8 (IL-8) secretion during a productive infection with chlamydia. Furthermore, expression of Toll-like receptor (TLR)-2 was required for IL-8 secretion from infected cells, whereas the effect of TLR4/MD-2 expression was minimal. Cell activation was dependent on infection with live, replicating bacteria, because infection with UV-irradiated bacteria and treatment of infected cells with chloramphenicol, but not ampicillin, abrogated the induction of IL-8 secretion. Finally, we show that both TLR2 and MyD88 co-localize with the intracellular chlamydial inclusion, suggesting that TLR2 is actively engaged in signaling from this intracellular location. These data support the role of TLR2 in the host response to infection with *C. trachomatis*. Our data further demonstrate that TLR2 and the adaptor MyD88 are specifically recruited to the bacterial or inclusion membrane during a productive infection with chlamydia and provide the first evidence that intracellular TLR2 is responsible for signal transduction during infection with an intracellular bacterium.

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The abbreviations used are: LGV, lymphogranuloma venereum; EB, elementary body; TLR, Toll-like receptor; LPS, lipopolysaccharide; IL-1, interleukin-1; GFP, green fluorescent protein; EGFP, enhanced GFP; TLR, Toll-like receptor; LPS, lipopolysaccharide; IL-1β, interleukin-1β; SHEC, human papillomavirus 16/E6E7 immortalized ectocervical epithelial cell line; HEK, human embryonic kidney cells; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

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that these interactions can distinguish between triacylated and diacylated lipid-modified lipoproteins/lipoproteptides, respectively. TLR3 is the receptor for double-stranded RNA and the synthetic analog polyinosine-polycytidylic acid (poly(IC)) (23); TLR5 recognizes flagellin (24); TLR9 recognizes bacterial cytosine-phosphate-guanosine (CpG) motifs in bacterial DNA (25, 26); and TLR7 and TLR8 recognize imidazoquinolines (27, 28) and synthetic GU-rich single-stranded RNA (29–31). No ligand has been identified yet for human TLR10.

Chlamydiae express a variety of ligands that could serve as potential TLR ligands. For example, chlamydial LPS has been extensively studied and found to have unique structural features, including an antigenically dominant disaccharide backbone (32–34) and a pentacylated lipid A structure with fatty acids of longer chain length with nonhydroxylated fatty acids ester-linked to the sugar backbone (35, 36). Unlike the LPS preparations from enteric Gram-negative bacteria, which are some of the most potent inducers of inflammation known, chlamydial LPS has been reported to be of low endotoxic activity (36–39). However, similar to the enteric LPS preparations, chlamydial LPS appears to utilize TLR4 for signaling, although it may be more dependent on membrane CD14 than has been reported for other species of LPS (38). A second important chlamydial antigen is the heat shock protein. When Kol and colleagues examined the proinflammatory activity of purified chlamydial HSP60 (cHSP60) preparations, they found that it resembled that of LPS, including the activation of the transcription factor NF-κB, activation of the p38 mitogen-activated protein kinase, and the induction of proinflammatory cytokines (40, 41). Several groups subsequently reported that cHSP60 could activate TLR2 and/or TLR4 (41–45). The chlamydia genome has also been shown to contain a number of known and hypothetical lipoproteins that would be potential TLR2 ligands.

Although a variety of chlamydial antigens might be recognized by individual TLRs, the role of specific TLRs during a productive infection with live bacteria remains unclear. Recently, Darville and colleagues (46) reported that the outcome of Chlamydia muridarum infection in TLR2-deficient mice was significantly altered in comparison to wild-type or TLR4-deficient mice. Although the course of genital tract infection was unchanged compared with that of wild-type and TLR4-deficient mice, the TLR2 knock-out strain had significantly lower levels of inflammatory mediators in genital tract secretions during the first week of infection, and there was a significant reduction in oviduct and mesosalpinx pathology at late time points. This suggested that TLR2 was the predominant receptor involved in the detection of and inflammatory response to chlamydia in the genital tract.

To further examine the role of specific TLRs in the host response to a productive chlamydia infection, we used an immortalized human cervical epithelial cell line, as well as transfected HEK293 cells to determine which members of the Toll-like receptor (TLR) family might be involved in the early events following attachment and invasion of chlamydia. We found that expression of MyD88 and TLR2 was required for cellular activation following infection with C. trachomatis, as measured by the secretion of the chemokine IL-8. In contrast, we found expression of TLR4/MD-2 had only a minor effect on IL-8 production by HEK293 cells infected with C. trachomatis. Cell activation required a productive chlamydia infection, and the use of UV-irradiated bacteria or treatment of infected cells with chloramphenicol following infection abrogated the response. In contrast, treatment with ampicillin, which effectively halts the differentiation of the organism, did not inhibit cytokine production, suggesting that bacterial replication and differentiation to at least the reticulate body phase was also required for cell activation. Finally, we found that TLR2 was tightly associated with the bacteria during the intracellular phase and that MyD88 was recruited along with TLR2 to the inclusion membrane. These data suggest that intracellular TLR2 is responsible for the initiation of signal transduction events during infection with C. trachomatis.

MATERIALS AND METHODS

Reagents—Phosphate-buffered saline and Trypsin-Versene Mixture (trypsin-EDTA) were obtained from BioWhittaker (Walkersville, MD), and fetal calf serum was obtained from HyClone (Logan, UT). Recombinant human IL-1β and tumor necrosis factor-α were purchased from R&D Systems (Minneapolis, MN). Synthetic lipopeptide Pam3Cys-Lip, which contains a tripalmitoyl-S-glyceryl cysteine at the amino terminus, was based on the amino-terminal sequence of the Neisseria gonorrhoeae F62 H.8/Lip protein (sequence CGGEKAAEAPAAEAS) (47) was purchased from EMC Microcollections (Tuebingen, Germany). LPS purified from Escherichia coli K235 was purchased from List Biologics (Campbell, CA). Contaminating endotoxin-associated proteins were removed by phenol re-extraction as described previously (48, 49). Ampicillin and chloramphenicol were purchased from Sigma-Aldrich.

Cell Culture—A human papillomavirus 16/E6E7 immortalized ectocervical epithelial cell line (ShEC) was engineered from human ectocervix collected as discarded surgical material at University Hospital, New Orleans, LA, with approval from the Institutional Review Board. The specimen was from a 35-year-old woman who had undergone hysterectomy for metrorrhagia. Ectocervix was dissected out from an area distant from the transformation zone, and the ectocervical margins were confirmed on frozen sections. Small pieces of tissue were placed epithelial side down in 6-well plates and cultured in keratinocyte serum-free medium (Invitrogen) supplemented with bovine pituitary extract and epidermal growth factor (as recommended and supplied by the manufacturer), and CaCl2 to a final concentration of 0.4 mM. Outgrowth of epithelial cells occurred within 10 days, and cells were then passaged twice before transduction with a retroviral vector (LXSN-16E6E7) packaged by the amphotrophic fibroblast line PA17 (50). Cells with integrated vector were selected by resistance to the neomycin analogue G418 and were passaged more than 20 times prior to experiments. The expression of the cytokeratin CK13 and lack of expression of secretory component confirmed the ectocervical derivation of the line. Cells were maintained in keratinocyte serum-free medium as described above with the addition of 20 µg of gentamicin per milliliter (Invitrogen).

Human embryonic kidney (HEK) 293 cell lines stably expressing fluorescent TLR chimeric proteins were a gift from Dr. Douglas Golenbock and have been previously described (51). The following cell lines were used in these studies: HEK293 expressing cyan fluorescent protein (CFP)- or yellow fluorescent protein (YFP)-tagged TLR2; HEK293 cells expressing YFP-tagged TLR4 and MD-2; and HEK293 cells expressing the empty vector pcDNA3. HEK293 cells expressing YFP-tagged TLR2 or YFP-tagged TLR4, with CFP-tagged MyD88, were engineered by transiently transfecting HEK/TLR2CFP or HEK/TLR4CFP/MD2 with the plasmid pcDNA3 containing CFP-tagged MyD88 (gift of Drs. Douglas Golenbock and Terje Espelid) using Gene Juice (Novagen, San Diego, CA) according to the manufacturer’s instructions. Cells were used for infection experiments on day 2 post-transfection. All HEK293 cell lines were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented with 10% FBS (HyClone) and 20 µg of gentamicin per milliliter.

Retroviral Transduction—ShEC cells stably expressing green fluorescent protein (GFP)-tagged MyD88 dominant-negative construct or GFP alone were engineered by retroviral transduction. The cDNA corresponding to amino acids 152–296 of human MyD88 (a kind gift of
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Dr. Marta Mizio (52) was PCR-amplified and cloned in the Xhol (5')-BamHI (3') sites of pEGFP-N1 (BD Biosciences-Clontech). The resulting cDNA encodes for a dominant negative version of MyD88 consisting of its TIR (Toll Interleukin 1 Resistance) domain, fused in-frame with the enhanced green fluorescent protein (ΔMyD88EGFP). The retroviral vector ΔMyD88EGFP was generated by subcloning the ΔMyD88EGFP cassette into the XhoI-NotI sites of the retroviral vector CLRCX − (53). Infectious retroviral particles were produced by transiently co-transfecting the packaging cell line 293gag/pol (~1 x 10^6 cells) with the retroviral plasmid (3 μg) and a plasmid encoding the vesicular stomatitis virus glycoprotein envelope protein (0.5 μg). The supernatant (3 ml) was then collected, filtered through a 0.22-mm nitrocellulose filter, and applied overnight to the target cells (5 x 10^5) in an equal amount of fresh medium containing 20 μg of Polybrene per milliliter (Sigma). Cells were allowed to recover for 2 days and re-infected. An identical backbone retrovirus encoding for the EGFP alone was used to construct a control cell line. Following transduction, cells were expanded and subjected to two rounds of positive selection using a MoFlo™ Ultra-High Speed Cell Sorter (DakoCytomation, Ft. Collins, CO) in enrichment mode to select for cells with high levels of GFP fluorescence.

**Bacterial Culture**—*C. trachomatis* L2/434/Bu was used for these studies (54, 55). Bacteria were routinely cultured in L.929 or McCoy cells. Cells were infected at an approximate multiplicity of infection (m.o.i.) of 0.5-1 before being centrifuged for 1 h at 37 °C. The cell culture medium was then replaced and cultured with Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FBS, 20 μg of gentamicin per milliliter, and 0.1 μg of cycloheximide per milliliter. Infected cells were harvested into sucrose-phosphate-glutamate buffer at 40 h post infection, sonicated, and stored at −80 °C until further use. Bacteria were subsequently titrated by the plaque assay (56, 57) using the L2 mouse fibroblast cell line. Chlamydia stocks tested negative for mycoplasma by PCR (58). To prepare UV-inactivated bacteria, chlamydia suspensions were placed under a 30-watt UV lamp at 30 cm for 30 min. Inactivation was confirmed by the failure to develop inclusions following infection of HeLa cells.

**Infection Studies**—All antibiotics were removed from cell culture medium 3 days prior to infection with chlamydia. For stimulation assays, cells were plated in 24-well tissue culture dishes at a density of 10^5 cells per well. Infection with *C. trachomatis* L2 was carried out by overlaying cells with an m.o.i. of 1-5. Other soluble stimuli were added as noted. Cells were incubated for 18–40 h at 37 °C (5%/5% CO2). Supernatant was harvested in 1.5-ml microcentrifuge tubes, spun for 5 min at 4 °C to pellet any cellular debris, transferred to clean tubes, and frozen at −80 °C. Culture supernatants were assayed for IL-8 using a DuoSet ELISA kit from R&D Systems, or IL-1β using an Eli-pair ELISA kit from Cell Sciences (Norwood, MA). Optical density was measured using a Bio-Kinetics microplate reader (Bio-Tek Instruments, Winsko, VT). All data points were assayed in triplicate and reported as the mean ± S.D.

**Confocal Microscopy Studies**—HEK293 cells were plated at a density of 7.5 x 10^5 cells per dish on glass bottom 35-mm tissue culture dishes (MatTek Corp., Ashland, MA) coated with 300 μg/ml Type 1 Rat Tail Collagen (BD Biosciences) 1 day prior to infection. For all confocal studies, infections were carried out by overlaying cells with an m.o.i. of 1-5. For antibody staining, cells were fixed and permeabilized with ice-cold methanol for 15 min and blocked for 5 min with PBS containing 5% FBS. Chlamydiae were stained using monoclonal antibody against chlamydia LPS (gift of Dr. You-Xun Zhang) followed by Alexa-647-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Cells were incubated for 20 min with each antibody, with three washes of PBS/5% FBS in between. Antibodies were used at a concentration of 10 μg/ml. Following the final antibody stain, cells were washed three times with PBS/5% FBS and mounted to a coverslip using SlowFade Light (Molecular Probes). Dishes were viewed under fluorescent confocal microscopy using a Zeiss LSM 510 Axiovert confocal laser scanning microscope equipped with an argon and helium laser, using a 63× objective. Dual color images were consecutively scanned using one active laser per scan.

**RESULTS**

**Epithelial Cells Secrete IL-8 in an MyD88-dependent Manner in Response to Productive Infection with C. trachomatis**—The epithelial cells that line the cervical mucosa are the first cells to contact sexually transmitted pathogens, as normally there are very few immune cells present in the cervicovaginal mucosa and lumen (59, 60). We previously reported that primary and immortalized cervical epithelial cells express a variety of TLRs, with the exception of TLR4 and the associated protein MD-2, rendering them unresponsive to LPS (61). However, despite the absence of LPS responsiveness, cells were readily activated by the Gram-negative pathogen *N. gonorrhoeae* in a predominantly TLR2-dependent fashion. We were interested in examining the response of cervical epithelial cells during the course of a productive infection with *C. trachomatis*, another common bacterial sexually transmitted infection. Ectocervical epithelial cells were infected with *C. trachomatis*, and supernatant was collected at 24 h post infection and assayed for the chemokine IL-8. As shown in Fig. 1A, we found a dose-dependent induction of IL-8 secretion over background following infection with chlamydia, with maximal stimulation occurring with an m.o.i. equal to 5.

To determine if this activation was dependent on TLRs, an ectocervical epithelial cell line stably expressing dominant-negative MyD88 was constructed. These cells were infected with *C. trachomatis*, and supernatant was again collected and assayed for IL-8. We found that IL-8 production was entirely dependent on MyD88 expression, suggesting the involvement of a TLR (Fig. 1B). Similar MyD88-dependent signaling was seen for stimulation with the synthetic lipopeptide Pam3Cys-Lip, a TLR2 ligand, while stimulation with tumor necrosis factor-α was MyD88-independent. Because the IL-1 receptor uses many of the same signaling proteins used by the TLRs, we wanted to exclude the possibility that the MyD88-dependent signaling was a result of feedback by IL-1. However, when the supernatant was assayed, we found no detectable IL-1β during the course of infection (data not shown).

**Cellular Responses to C. trachomatis Require Expression of TLR2 and the Presence of Live, Replicating Bacteria**—Given the important role of TLR2 in cervical epithelial cell responses to a variety of microbial ligands, we hypothesized that TLR2 would play a major role in epithelial cell responses to *Chlamydia*. To test this, we used transfected HEK293 epithelial cells, which fail to express their own endogenous TLR2 or TLR4, thus enabling us to isolate TLR2 and TLR4 function. HEK293 cells stably transfected with TLR2, TLR4/MD-2, or the control neomycin resistance cassette, were infected with *C. trachomatis* at an m.o.i. of 5. Supernatant was collected at 24 h and assayed for IL-8. We found predominantly TLR2-dependent IL-8 secretion, with minimal TLR4-dependent activity (Fig. 2). This was somewhat surprising considering that chlamydiae are known to express TLR4 ligands, including LPS and hsp60. Given that the response was predominantly TLR2-dependent, and that TLR4/MD-2 appeared to be absent from cervical epithelium (61), we concluded that TLR2 would be the primary receptor involved in the recognition of chlamydia during genital tract infection and focused our remaining studies on TLR2. We next examined the time course of
chlamydia-induced cell activation, looking specifically at TLR2-dependent signaling. HEK293 cells stably expressing TLR2 were infected with *C. trachomatis* and assayed over time for IL-8 production. As shown in Fig. 3, we found that IL-8 production increased over time during chlamydia infection, peaking at 40 h post infection. Later time points were not tested because of concern over how cell lysis from the intracellular inclusions might affect the assay. This stands in contrast to the activation with the soluble ligand Pam3Cys-Lip, which peaked much earlier at 16 h.

We next wanted to determine if cellular activation absolutely required a productive chlamydia infection with live, replicating bacteria. We first examined the effect of incubating cells with an identical m.o.i. of either UV-irradiated EBs or live EBs and found that UV-irradiated bacteria failed to activate IL-8 production (data not shown). We repeated the experiment using antibiotics to either kill the bacteria or inhibit the chlamydia growth cycle. Cells were infected with live EBs and, 2 h post infection, cells were treated with either chloramphenicol or ampicillin. At 40 h, cells were examined for the presence of inclusions, and supernatant was assayed for IL-8. The antibiotic treatments had the expected results, with chloramphenicol treatment inhibiting the development of chlamydial inclusions, and ampicillin treatment resulting in the development of large inclusions with the characteristic irregularly shaped RB bacterial forms, as has been reported in the literature (62, 63) (data not shown). When assayed, supernatant from the chloramphenicol-treated cells failed to demonstrate up-regulation of IL-8, whereas both the untreated and ampicillin-treated cells did (Fig. 4). This suggested to us that cellular activation required both internalization and replication of the chlamydia.

**TLR2 and MyD88 Are Recruited to the Inclusion Membrane of Chlamydia-infected Cells**—Based on our biological data, we hypothesized that the TLR2-dependent cell activation was induced by the
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FIGURE 4. C. trachomatis activation of TLR2 is inhibited by chloramphenicol but not ampicillin. HEK293 cells stably expressing TLR2 were plated in 24-well dishes and infected with C. trachomatis serovar L2 at the m.o.i. indicated. 2 h after addition of bacteriawater, chloramphenicol (100 μg/ml), or ampicillin (10 μg/ml) was added to the indicated wells. Synthetic lipopeptide Pam3Cys-Lip (P3C Lip; 25 ng/ml) was used as a control. Supernatant was collected at 40 h and assayed for IL-8 by ELISA. Data are reported as the mean ± S.D. of triplicate wells. Shown above is a representative of two independent experiments.

 Cells show a distribution of TLR2 (green) primarily at the cell surface, with some expression in the peri-nuclear region likely representing accumulation in the Golgi. At 16 h post infection, multiple small inclusions, most likely containing replicating RBs, can be seen in the infected cells. TLR2 (green) can be seen co-localizing with the chlamydia (red) at one end of the bacterial forms (seen as yellow color, middle row, Fig. 6). At 24 h post infection, the smaller inclusions have now fused to form one large inclusion localized in the peri-nuclear region. Here, marked co-localization of the TLR2 and chlamydia can again be seen at the periphery of the inclusions (seen as yellow color, bottom row, Fig. 6).

DISCUSSION

C. trachomatis is an obligate intracellular organism that provides a unique opportunity to study the interaction of host cell proteins with an invasive pathogen. The primary purpose of our work was to examine the role of TLRs in the response of epithelial cells to a productive chlamydial infection. Our initial studies focused on identifying the specific TLRs and adaptor proteins that were important in recognition of and response to C. trachomatis. As others have reported (46, 65), we found a requirement for both TLR2 and MyD88 in cellular activation during chlamydial infection. Furthermore, our data demonstrate that cells are activated not simply by ligands expressed at the surface of the elementarybody, but by a ligand or ligands that are expressed during an active and productive intracellular infection. Although the surface of even irradiated chlamydia elementary bodies are likely to express LPS, lipoproteins, and other potentially biologically active structures, our data suggest that this surface alone is not sufficient to efficiently activate cells. It is possible that the bacteria must reach a critical mass to activate the TLR2 pathway and that this mass cannot be reached when cells are infected with irradiated bacteria or treated with bacterial antibiotics. It is also possible that the specific ligand that is activating TLR2 is only produced or only accessible to the receptor when RB forms are actively dividing within the inclusion. In support of this is the observation that ampicillin treatment, which essentially halts the growth cycle before the conversion of RBs to EBs, does not abrogate the cytokine response to infection. Evidence to suggest that TLR2 and MyD88 are recruited to the inclusion or bacterial surface would support either of these explanations. The actual ligand or ligands that are responsible for the TLR2-dependent activation remain unknown.

Confocal microscopy is a powerful tool for examining the interaction between TLRs and microbial pathogens. Because endogenous protein expression can often be too low for visualization by microscopy, transfected cell lines expressing fluorescent chimeric constructs have become an essential component of studies on receptor trafficking, in particular if live cell imaging is to be performed. Our hypothesis that intracellular TLR2 is capable of signaling is not without precedent. It has been reported that TLR2 traffics to phagocytic vacuoles containing zymosan particles, leading some researchers to suggest that it could “sample” the contents of vacuolar compartments (66). What sets chlamydiae apart from other intracellular pathogens is the unique chlamydial inclusion, which neither fuses with lysosomes nor acidifies (67). The subcellular localization of the chlamydial inclusion in the Golgi region provides an important niche for the organism to intercept sphingoglycolipid-containing vesicles (68, 69). It might also provide an immunologically hidden site for the bacteria to evade immune detection. The active recruitment of TLR2 with MyD88 to the intracellular chlamydiae suggests that this is, in fact, not the case. However, the delay in cell activation relative to the initiation of infection might provide a protected window of time for the organism to replicate and prepare for secondary infection of neighboring cells, before the immune system can

chlamydia replicating within the inclusion, and that intracellular TLR2 might possibly be responsible for the sensing. The TLR2- and TLR4-transfected cell lines used for the biological assays above express a fluorescent-tagged TLR protein fused at the carboxyl terminus to either a yellow or cyan fluorescent protein. These chimeric proteins are thus fully functional, easy to detect under fluorescent microscopy, and allow us to identify intracellular proteins during imaging of live cells. Although co-localization of TLR2 with the bacteria would likely be required for intracellular TLR activation, it would not be sufficient evidence that the receptor was actively signaling from this site.

To examine receptor activation, we looked at infected cells for the intracellular localization of TLR2 relative to that of the adaptor protein MyD88, which is shared by all members of the TLR/IL-1 family with the exception of TLR3 (64). As a control, we made comparisons to TLR4-transfected cells, which were not significantly activated during infection. In uninfected cells, there is no co-localization of TLR2 and MyD88. However, at 24 h post-infection (Fig. 5B), TLR2 (red) and MyD88 (green) can be seen co-localizing at the inclusion membrane (yellow), consistent with activation of the intracellular TLR2. In contrast, when TLR4 localization was examined in infected cells, we found some TLR4 expressed at the inclusion membrane, but co-localization of MyD88 with the TLR4 was not seen (Fig. 5D).

We wanted to examine the co-localization of TLR2 and chlamydia in more detail. HEK293 cells stably expressing CFP-tagged TLR2 were infected with chlamydia. At an early (16 h) or late (24 h) time point following infection, cells were fixed and permeabilized and the chlamydiae were identified using an anti-chlamydia LPS primary antibody counterstained with Alexa-647. As shown in Fig. 6 (top row), uninfected...
mount an adequate response. While it would appear from our data that the TLR2 localizes to the bacterial membrane, this cannot be stated with certainty based on these images alone. The tight association between the replicating RBs, which are generally located at the periphery of the inclusion, and the inclusion membrane itself makes it difficult to differentiate the two surfaces at this level. Further studies to determine precisely the location of this interaction relative to the chlamydial surface and the inclusion membrane will be required to resolve this question. For example, electron microscopy might better distinguish the RB and EB forms from the inclusion membrane. This distinction is important in hypothesizing the potential TLR2 ligands that might be biologically relevant in this signaling event. For example, electron microscopy might better distinguish the RB and EB forms from the inclusion membrane. This distinction is important in hypothesizing the potential TLR2 ligands that might be biologically relevant in this signaling event. For example, a cursory review of the chlamydia genome reveals a number of lipoproteins and hypothetical lipoproteins, which could be considered potential TLR2 ligands. However, there are likely to be fewer ligands that are specifically localized to the inclusion membrane. Furthermore, considering the complicated developmental cycle of chlamydia, one must consider the possibility that the TLR2 ligand is developmentally regulated. This might also be important in trying to identify the specific ligand.

Another unanswered question is the mechanism by which TLR2 finds its way to the inclusion or bacterial membrane. In resting cells, at least, TLR2 appears to be expressed primarily at the cell surface (70). Thus, it would be expected that TLR2 might be internalized with the EB during uptake by the host cell. However, any receptors that are carried

infection, live cells were viewed under fluorescent confocal microscopy using a 63× objective. Groups A and C depict uninfected cells, whereas groups B and D depict chlamydia-infected cells. Within each group, the top left and right images were acquired by consecutive scanning; the bottom right image displays the overlay of the two previous images; the bottom left image shows the phase-contrast view. "I" denotes the characteristic chlamydial inclusion that can be visualized in the phase-contrast image. Co-localization between the TLR (red) and MyD88 (green) can be seen as yellow color at the chlamydial inclusion in the TLR2- but not the TLR4-infected cells.
along with the cell membrane to form the early inclusion membrane would be rapidly diluted during bacterial replication and growth of the inclusion surface area. The continued association between TLR2 and the inclusion membrane but actively engaged in cell signaling from that site. Whether there is a requirement for an additional cell signal to recruit TLR4 would suggest that the TLR2 is not simply passing by the inclusion membrane but actively engaged in cell signaling from that site. Whether cell activation and TLR2/MyD88 recruitment are specific to the RB or EB phase of the bacteria. Further studies will be required to determine the need to reach a critical mass of organisms to efficiently activate the immune system. It might also reflect expression of a developmentally regulated ligand with which TLR2 is interacting. Because the chlamydial life cycle is not synchronized, we cannot determine from our data whether cell activation and TLR2/MyD88 recruitment are specific to the RB or EB phase of the bacteria. Further studies will be required to better understand both the biology and immunology of this interaction, as well as the role this plays in disease.

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