Toll-Like Receptor-2, but Not Toll-Like Receptor-4, Is Essential for Development of Oviduct Pathology in Chlamydial Genital Tract Infection

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*J Immunol* 2003; 171:6187-6197; doi: 10.4049/jimmunol.171.11.6187

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Toll-Like Receptor-2, but Not Toll-Like Receptor-4, Is Essential for Development of Oviduct Pathology in Chlamydial Genital Tract Infection

Toni Darville,*† Joshua M. O’Neill,* Charles W. Andrews, Jr., ‡ Uma M. Nagarajan,† Lynn Stahl,*§ and David M. Ojcius§

The roles of Toll-like receptor (TLR) 2 and TLR4 in the host inflammatory response to infection caused by Chlamydia trachomatis have not been elucidated. We examined production of TNF-α and IL-6 in wild-type TLR2 knockout (KO), and TLR4 KO murine peritoneal macrophages infected with the mouse pneumonitis strain of C. trachomatis. Furthermore, we compared the outcomes of genital tract infection in control, TLR2 KO, and TLR4 KO mice. Macrophages lacking TLR2 produced significantly less TNF-α and IL-6 in response to active infection. In contrast, macrophages from TLR4 KO mice consistently produced higher TNF-α and IL-6 responses than those from normal mice on in vitro infection. Infected TLR2-deficient fibroblasts had less mRNA for IL-1, IL-6, and macrophage-inflammatory protein-2, but TLR4-deficient cells had increased mRNA levels for these cytokines compared with controls, suggesting that ligation of TLR4 by whole chlamydiae may down-modulate signaling by other TLRs. In TLR2 KO mice, although the course of genital tract infection was not different from that of controls, significantly lower levels of TNF-α and macrophage-inflammatory protein-2 were detected 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. TLR4 KO mice responded to in vivo infection similarly to wild-type controls and developed similar pathology. TLR2 is an important mediator in the innate immune response to C. trachomatis infection and appears to play a role in both early production of inflammatory mediators and development of chronic inflammatory pathology. The Journal of Immunology, 2003, 171: 6187–6197.

In the realm of infectious diseases, it has often been observed that an overly aggressive inflammatory host response can be more problematic than the infection that initiated it. This is certainly true in the case of genital tract infection with Chlamydia trachomatis, where the pathology that leads to fallopian tube inflammation, scarring, and infertility is the result of a robust host inflammatory response. C. trachomatis is a Gram-negative obligate intracellular bacterium that can initiate a variety of immune system responses from infected hosts. In mice, infection with the mouse pneumonitis strain of C. trachomatis as well as its role in inflammation and the resulting pathological sequelae that occur (5–10). The relatively new discovery of the involvement of Toll-like receptors (TLRs) (11) in the innate host response has prompted investigations of these receptors as potential regulators of the response to chlamydiae.

TLRs are a family of proteins that share homology with the Toll antimicrobial proteins of Drosophila (12). These receptors are found primarily on mammalian innate immune cells, such as macrophages and dendritic cells, but are also expressed on many epithelial cells. TLRs act as pattern recognition receptors that enable cells to recognize pathogen-associated molecular patterns (PAMPs). This sheds light on the previously unappreciated specificity afforded to the innate immune system, whereby the TLRs allow for immediate recognition of conserved bacterial, viral, and fungal elements (13, 14). Engagement of these receptors can lead to activation of phagocytes and, through activation of the transcription factor NF-κB, production of inflammatory cytokines such as TNF-α and IL-6 (15–17). Of the 10 currently described TLRs, TLR2 and TLR4, along with their ligands, are perhaps the best understood in terms of innate responses to bacteria. TLRs recognize a variety of PAMPs. TLR4 is the signal-transducing receptor for LPS (18); it is aided by two accessory proteins, CD14 and MD-2 (19). TLR2 is involved in the recognition of a broad range of microbial products, including peptidoglycan (PGN) from Gram-positive bacteria (20), bacterial lipoproteins (21), mycobacterial cell wall lipoarabinomannan (22), and yeast cell walls (23).

Chlamydia has several cell wall and outer membrane components that may serve as PAMPs recognized by TLRs. Chlamydial LPS (24) and chlamydial heat shock proteins (HSPs) (25–27) are ligands for the TLR4 receptor. However, recent papers describe TLR4-independent cytokine production from Toll-like receptors in response to chlamydiae.

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Received for publication July 18, 2003. Accepted for publication September 29, 2003.

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This work was supported by Public Health Service Grant AI054624 from the National Institutes of Health and by the Horace C. Cabe Foundation and the Bates-Wheeler Foundation, Arkansas Children’s Hospital Research Institute and University of Arkansas Medical Sciences, and the Fondation pour la Recherche Médicale.

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Abbreviations used in this paper: MoPn, mouse pneumonitis agent of Chlamydia trachomatis; EB, elementary body; HSP, heat shock protein; CHSP60, chlamydial heat shock protein 60; KO, knockout; MIP-2, macrophage-inflammatory protein-2; MOI, multiplicity of infection; PGN, peptidoglycan; PAMP, pattern-associated molecular pattern; TLR, Toll-like receptor; IFU, inclusion-forming unit.
dominant role for TLR2 vs TLR4 in the recognition process of C. pneumoniae (also known as Chlamydiophila pneumoniae) (30).

The effect of TLR2-deficiency on the macrophage response to C. trachomatis infection and the effect of a deficiency of either TLR4 or TLR2 have not been described in an in vivo model of Chlamydia infection. We examined cytokine release in vitro from peritoneal macrophages and primary fibroblasts obtained from mice deficient in the genes for TLR4 and TLR2 to determine the relative contribution of these two pattern recognition receptors in the inflammatory cell response to C. trachomatis. In addition, we determined the roles of TLR2 and TLR4 in the in vivo response to chlamydial genital tract infection by examining the course of chlamydial infection, the local inflammatory response, and chronic histopathology in infected mice deficient for either TLR2 or TLR4.

Materials and Methods

Animals
C57BL/6 mice, C57BL/10 mice, and mice homozygous for Tlr2−/− (C57BL/10ScCr) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice homozygous for Tlr4−/− were kindly provided by S. Akira (Osaka, Japan) and were bred in our own animal facility. Female mice homozygous for Tlr2−/− were bred with C57BL6 mice, and the F1 progeny (heterozygotes) were used as controls for TLR2 knockout (KO) experiments. The mice were between 6 and 10 wk old at the time of purchase and between 8 and 16 wk of age for different experiments. Wild-type and control groups were age matched for all experiments. Mice were given food and water ad libitum in an environmentally controlled room with a cycle of 12 h of light and 12 h of darkness. All animal experiments were approved by the University Institutional Animal Care and Use Committee.

Reagents and bacteria

Purified LPS of Escherichia coli serotype 011:B4 was purchased from InvivoGen (San Diego, CA). Purified PGN, isolated from the sonicated cell wall of Streptococcus pyogenes, group A, D58, was purchased from Lee Labs (Grayson, GA). The MoPn agent of C. trachomatis (30), also known as Chlamydia muridarum (30), was grown in Mycoplasma-free McCoy or HeLa229 cells, and EBs were harvested from infected cells by centrifugation as previously described (31).

Isolation of murine peritoneal macrophages

Three days before murine peritoneal macrophages were harvested, mice were given a 1-ml i.p. injection of 3% thioglycollate medium. To harvest macrophages, mice were euthanized by cervical dislocation, and their peritoneal cavities were washed three times with 10 ml of macrophage culture medium. Peritoneal macrophages were plated into 24-well culture plates containing coverslips at a concentration of 1 × 10⁶ macrophages/well. Cells were washed with fresh medium after 60 min to remove nonadherent cells.

In vitro infection of macrophages

Three days after the murine macrophages were harvested, in vitro infections were performed. Varying concentrations of stock MoPn or UV-inactivated MoPn from the same culture stock were added to macrophage culture medium to allow for different multiplicities of infection (MOIs), such that an MOI of 1 represented 1 inclusion-forming unit (IFU) per macrophage. UV-inactivated EBs were prepared by exposing purified EBs to UV light in a laminar flow hood for 3 h. After UV inactivation, the EBs were checked to confirm that no infectious organisms were present. MOIs used were 0.5, 1, 2, 5, and 10. One milliliter of infection medium or, as a negative control, medium containing a lysate of uninfected McCoy cells, was placed over the macrophage monolayer in each well, and the plates were centrifuged (1800 × g, 60 min, 35°C). Cells plated with live EBs were then washed with macrophage medium to remove any excess EBs, and 1 ml of fresh macrophage medium was placed in each well. Finally, the plates were incubated for 75°C for 5 min. After 24 and 48 h after infection, 70 µl of supernatant were removed from the appropriate wells and frozen at −70°C until cytokine assays could be performed. At these same time points, coverslips were removed after aspiration of medium, fixed with methanol for at least 30 min, and stained with Pathfinder fluorescein-conjugated murine anti-chlamydial MAb according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Macrophage monolayers were viewed with an Olympus microscope (Olympus, Melville, NY) to evaluate inclusion formation.

Establishment of murine lung fibroblasts and in vitro culture with MoPn

Lung fibroblasts from C57BL/6, C57BL/10ScCr, and Tlr2−/− mice were isolated as previously described (32) and cultured for 5 to 6 days at 37°C in an atmosphere of 5% CO₂ in RPMI 1640 containing supplemented with 20% heat-inactivated FBS (Life Technologies, Gaithersburg, MD) and 2 mM t-glutamine. After the cells reached confluence, culture medium was aspirated, and the cells were infected with C. trachomatis at an MOI of 0.25, 0.5, or 0.5 for 24 h.

Real time PCR for IL-1β, IL-6, and macrophage inflammatory protein-2 (MIP-2) mRNA from murine fibroblasts

RNA from uninfected and infected fibroblasts was isolated using an RNeasy kit (Qiagen, Chatsworth, CA) following the manufacturer’s instructions. Total RNA was converted to cDNA by standard reverse transcription of virus reverse transcriptase in the macrophage’s buffer (Roche Diagnostics, Meylan, France). Quantitative PCR was performed with 1/20 of the cDNA preparation in the GeneAmp 5700 (Applied Biosystems, Foster City, CA) (33) in 25 µl with Taq polymerase (Hot StarGold enzyme, 0.025 U/ml) in Taqman buffer (Eurogentec, Brussels, Belgium) containing 5 mM MgCl₂ and 200 µM concentrations of each dNTP. cDNA was amplified using 0.8 µM concentrations of each specific sense primer and 0.8 µM antisense primers. We also used 0.4 µM concentrations of the fluorogenic oligonucleotides specific for the gene segments in which a reporter fluorescent dye on 5’ (FAM) and a quencher dye on 3’ (TAMRA) were attached. Real time PCR was conducted at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s. The specific activities of cDNA from different cytokines were compared with β-actin and normalized to uninfected wild-type fibroblast responses by the comparative Ct method as described by the manufacturer. Forward primers, reverse primers, and fluorescent probes for murine β-actin, IL-1β, MIP-2, and IL-6 are listed in Table I.

RT-PCR for gelant tract TLR2 and TLR4 mRNA from upper and lower genital tract tissues

Mice in groups of three were euthanized before (day −1) and at different time points after (days 7, 14, and 21) infection with MoPn. The upper (uterus horns and oviducts) and lower (upper vagina and multilocular intravaginal) genital tracts were removed from each mouse and homogenized individually. The homogenates were then processed using an RNeasy kit (Qiagen) according to the manufacturer’s instructions to isolate RNA from each sample. The total RNA concentration and purity were evaluated by OD readings at 260 and 280 nm. DNase treatment was performed in a 20-µl reaction mixture containing PCR buffer and 1.5 mM MgCl₂ (Applied Biosystems), 3.5 mM MgCl₂, 1 µM each (dNTPs, 1 µM oligo(dT) primer, 1 µM random hexamer, 0.5 µl of RNasin (Promega, Madison, WI), 0.5 µl RNase-free DNase (Promega), and 1 µg of RNA from each sample. Samples were placed in a PCR block at 37°C for 15 min followed by 70°C for 10 min. Then, 0.5 µl of reverse transcriptase (SuperScript II; Invitrogen, Carlsbad, CA) was added to cause the reverse transcription reaction at 25°C for 10 min, 42°C for 40 min, and 95°C for 5 min. A control reaction containing all components except reverse transcriptase was conducted for each RNA sample to check for contaminating nucleic acids. cDNAs were amplified by using the Bioline PCR system (Canton, MA) in a 50-µl reaction mixture containing one-twentieth of the cDNA generated from reverse transcription reaction, 1× PCR buffer, 1.5 mM MgCl₂, 1 µM each (dNTPs, 1 µM forward and reverse primers, and 0.5 U Taq polymerase. The sequences of the primers used for murine β-actin, TLR2 and TLR4 are listed in Table I. PCR conditions for all primers were as follows: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Marine infection with Chlamydia and measurement of chlamydial shedding

Mice received 2.5 mg of Depo-Provera (Pfizer, New York, NY) in 0.1 ml of saline (medroxyprogesterone acetate; Upjohn, Kalamazoo, MI) s.c. 7 days before vaginal infection. The mice were infected by placing 30 µl of 250 µg/microscope, 10 mM sodium phosphate, 5 mM t-glutamic acid (pH
Table I. Forward primers, reverse primers, and fluorescent probes used for real time PCR; forward and reverse primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
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<tr>
<td>β-Actin</td>
<td>5'-AGGAGGAATCAGCTGGTGG-3'</td>
<td>5'-GGTTAGGGTGTGCGTATG-3'</td>
<td>TAMRA-3'</td>
</tr>
<tr>
<td>MIP-2</td>
<td>5'-CACTGCCGCATCCTCTTCCTCCC-3'</td>
<td>5'-GATCCACACTCTCCAGCTGCA-3'</td>
<td>TAMRA-3'</td>
</tr>
<tr>
<td>IL-1</td>
<td>5'-CAACCAACAAGTGATATTCTCCATG-3'</td>
<td>5'-GATCCACACTCTCCAGCTGCA-3'</td>
<td>TAMRA-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-TCCTACCCCAATTTCCAATGC-3'</td>
<td>5'-TGAATTGGATGGTCTTGGTCCT-3'</td>
<td>TAMRA-3'</td>
</tr>
<tr>
<td>TLR2</td>
<td>5'-TGCAAGTACGAACTGGACTTCT-3'</td>
<td>5'-CCAGGTAGGTCTTGGTGTTCATT-3'</td>
<td>TAMRA-3'</td>
</tr>
<tr>
<td>TLR4</td>
<td>5'-AAGATACACCAACGGCTCTGAA-3'</td>
<td>5'-CCAGGTAGGTCTTGGTGTTCATT-3'</td>
<td>TAMRA-3'</td>
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Collection of genital tract secretions for cytokine analysis

Genital tract secretions were collected from mice on multiple days throughout the course of infection and analyzed by ELISA for various cytokines of interest. At intervals before and after infection, an aseptic surgical sponge (ear wicks, 2 × 5 mm) (DeRoyal, Powell, TN) was inserted into the vagina of an anesthetized mouse and retrieved 30 min later. The sponges were held at −70°C until the day of the cytokine assay. Each sponge was placed in a Spin-X microcentrifuge tube (Fisher Scientific, Pittsburgh, PA) containing a 0.2-μl cellulose acetate filter, incubated in 300 μl of sterile PBS with 0.5% BSA and 0.05% Tween 20 for 1 h on ice, and then centrifuged for 5 min. Spin-X filters were preblocked with 0.5 ml of sterile PBS with 2% BSA and 0.05% Tween 20 for 30 min at 25°C, centrifuged, and washed twice with 0.05 ml of sterile PBS. Samples were kept on ice and promptly loaded into an ELISA plate prepared for a specific cytokine assay.

Detection of cytokines

Genital tract sponge eluates and macrophage culture supernatants were assayed individually for cytokine activity by ELISA using commercial cytokine ELISA kits for TNF-α, IL-6, IFN-γ, and the chemokine, MIP-2 (R&D Systems, Minneapolis, MN).

Histopathology

Mice were sacrificed on days 7 and 35 after infection, and the entire genital tract was removed en bloc, fixed in 10% buffered formalin, and embedded in paraffin. Longitudinal 4-μm sections were cut, stained with H&E, and evaluated by a pathologist blinded to the experimental design. Each anatomic site (excocervix, endocervix, uterine horn, oviduct, and mesosalpinx) was assessed independently for the presence of acute inflammation (neutrophils), chronic inflammation (lymphocytes), plasma cells, and fibrosis. Laminar distention of the uterine horns and dilatation of the oviducts were graded from 1 to 4, with grade 4 representing severe hydrosalpinx. Right and left uterine horns and right and left oviducts were evaluated individually. A four-tiered semiquantitative scoring system was used to quantify the inflammation and fibrosis: 0 = normal; 1+ = rare foci (minimal presence) of parameter; 2+ = scattered (1–4) aggregates or mild diffuse increase in parameter; 3+ = numerous aggregates (>4) or moderate diffuse or confluent areas of parameter; 4+ = severe diffuse infiltration or confluence of parameter.

Evaluation of serum Ab responses

Sera from mice were collected by retro-orbital blood sampling at the time of sacrifice and stored at −20°C until analyzed by ELISA as previously described (35). Preimmune sera were used as negative controls. The titer for individual mice was determined as the highest serum dilution with an OD value greater than that of the control wells.

Statistics

The ANOVA plus post hoc test was used to analyze differences in cytokine production among various in vitro groups. Statistical comparisons between the murine strains for level of infection and cytokine production over the course of infection were made by a two-factor (days and murine strain) ANOVA with post hoc Tukey test as a multiple comparison procedure. The Wilcoxon rank sum test was used to compare the duration of infection in the respective strains over time. The Kruskal-Wallis one-way ANOVA on ranks was used to determine significant differences in the pathological data between groups. The χ2 test for determination of significant differences in sample proportions was used to compare frequencies of pathological findings between specific groups. SigmaStat software was used (SPSS Science, Chicago, IL).
Results

TNF-α and IL-6 release in response to MoPn infection is markedly diminished in TLR2-deficient murine macrophages

Macrophages play a key role in early innate immune responses and produce proinflammatory cytokines such as TNF-α and IL-6 when activated. We determined the production of these cytokines by peritoneal macrophages from TLR2 KO and heterozygous littermates after in vitro infection with MoPn. Macrophages lacking TLR2 produced significantly decreased amounts of TNF-α and IL-6 in response to MoPn infection (Fig. 1). This provides evidence that recognition of chlamydiae by TLR2 and subsequent signaling requires active infection of the macrophage as UV-inactivated EBs bind to and are ingested by macrophages. Although decreases in TNF-α were not as strong at an MOI of 5, marked decreases in IL-6 were seen at all MOIs. Also, in all experiments, most of the cytokine production occurred during the first 24 h of infection, with levels detected at 48 h being minimally increased over the earlier time point (data not shown).

Macrophages release TNF-α and IL-6 in response to MoPn infection independently of TLR4

Identical experiments were then repeated using TLR4-deficient macrophages. Unexpectedly, when macrophages from TLR4 KO and control mice were used, significantly higher TNF-α and IL-6 levels were detected from the TLR4 KO macrophages compared with C57BL/10 controls at all MOIs tested (1, 2, and 5) (Fig. 2). UV-inactivated EBs elicited very low cytokine responses from both TLR4 KO and normal macrophages, again indicating that stimulation of murine macrophages by intact EBs requires active chlamydial infection. Thus, more cytokines are secreted in the absence of TLR4 than in its presence, suggesting that TLR4 ligation may down-modulate signaling through another TLR (presumably TLR2) or that expression of TLR2 and/or other TLRs may be higher in the absence of TLR4.

Lower mRNA accumulation for IL-1β, IL-6, and MIP-2 occurs after MoPn infection of lung fibroblasts from TLR2 KO mice compared with controls, but higher mRNA accumulation occurs in fibroblasts from TLR4 KO mice

A 24-h infection of primary fibroblasts with C. trachomatis at an MOI of 0.5 led to a large increase in transcription of the IL-1β, MIP-2, and IL-6 genes. Significantly lower levels of transcription for the three cytokine genes were consistently found in TLR2-deficient fibroblasts infected at an MOI of 0.5 or 0.25, compared with wild-type fibroblasts infected with the same MOIs (Fig. 3). These results are in agreement with the decreased secretion of TNF-α and IL-6 observed for TLR2-deficient macrophages, compared with wild-type macrophages.
Similarly, higher levels of cytokine gene transcription were found in TLR4-deficient fibroblasts after a 24-h infection with *C. trachomatis* compared with wild-type fibroblasts infected with the same MOIs (Fig. 4). Given that infected TLR4-deficient macrophages also secrete higher concentrations of TNF-α and IL-6 than infected wild-type macrophages, these results suggest that TLR4 may decrease cytokine production by cells infected with live *C. trachomatis*, regardless of the cell type used for infection.

mRNAs for both TLR2 and TLR4 are present in the upper and lower genital tract before and during infection

Previous studies with primary human epithelial cells derived from normal human vagina, ectocervix, and endocervix revealed the presence of mRNA for TLR2, but an absence of TLR4 message (36). Homogenized whole tissues from both the upper and lower genital tracts of mice before and after infection were analyzed by RT-PCR for mRNA for both TLR2 and TLR4 receptors. Positive cDNA bands for both TLR2 and TLR4 were found in three of three mice tested on day −1, 7, 14, and 21 of infection (Fig. 5). Thus, although TLR4 may not be present in epithelial cells of the lower genital tract, it is easily detected in whole genital tract tissue from both the upper and lower genital tracts. We cannot make any conclusion as to the relative levels of gene expression for these two TLRs in the genital tract during chlamydial infection because the methodology was purely qualitative.
TNF-α and MIP-2 levels are significantly decreased in genital tract secretions of TLR2 KO mice after infection, whereas IFN-γ and IL-6 are only marginally reduced and TLR4 KO mice have responses similar to infected controls

Given the presence of both TLR2 and TLR4 in the murine genital tract, we used daily genital tract sponges to examine the local production of TNF-α, MIP-2, IFN-γ, and IL-6 for the first 10 days of infection. As seen in Fig. 6, compared with controls, TLR2 KO mice had significantly reduced levels of TNF-α (Fig. 6A) and MIP-2 (Fig. 6C) detected in their genital tract secretions. However, although IFN-γ and IL-6 levels were also reduced, the decrease was not significant (Fig. 6, B and D).

TLR4-deficient mice produced similar amounts of inflammatory mediators to controls early during infection (Fig. 7). Thus, despite the increased cytokine levels detected in vitro from Chlamydia-infected TLR4-deficient macrophages and fibroblasts, increased levels were not seen with in vivo infection in TLR4 KO mice.

Course of MoPn infection is equivalent in TLR2 KO, TLR4 KO, and wild-type mice

Fig. 8 shows that the course of infection as enumerated from vaginal swabs was essentially identical in all groups of mice regardless of the presence or absence of TLR2 (Fig. 8A) or TLR4 (Fig. 8B). Thus, despite the marked decrease in levels of TNF-α and MIP-2 detected in genital tract secretions in TLR2 KO mice, they resolved the infection as efficiently as controls.

Infected TLR2 KO mice display less pathology than wild-type mice

Despite their similar course of infection, chronic oviduct pathology was markedly reduced in TLR2 KO mice compared with infected heterozygous litters (Fig. 9). Histologically, none of the infected TLR2 KO mice developed hydrosalpinx or severe dilatation of their oviducts. Forty percent of the oviducts in the control mice were severely dilated, with moderate dilatation in another 50%. The majority (75%) of the oviducts from the TLR2 KO mice were only mildly dilated. On day 35 of infection, although inflammatory cells were noted throughout the genital tracts of both groups, acute inflammatory cells were significantly decreased in the TLR2 KO mice in the oviduct (Fig. 10A) and mesosalpinx (Fig. 10B). Specifically, TLR2 KO mice showed less oviduct dilatation, fewer

FIGURE 6. TLR2 is important for TNF-α and MIP-2 responses with in vivo Chlamydia infection, but IFN-γ and IL-6 responses are minimally affected by TLR2 deficiency. Mean (± SEM) TNF-α (A), IFN-γ (B), MIP-2 (C), and IL-6 (D) levels in genital tract secretions of TLR2+/− and TLR2−/− mice during the first 10 days of infection with MoPn of C. trachomatis. Data represent the combined results of two separate experiments (n = 5 per strain per experiment). Each sample was run in duplicate. \( p < 0.005 \) by two-factor ANOVA for TNF-α (A) and MIP-2 (C) between TLR2+/− vs TLR2−/− groups.
acute inflammatory cells and less mesosalpingeal inflammation and fibrosis than controls.

The genital tract tissues from TLR4 KO mice exhibited pathology equal to control mice. Inflammatory cell infiltration and degree of oviduct dilatation were no different from infected wild-type mice sacrificed on the same day (data not shown).

A predominance of IgG2a was detected in serum of infected controls, TLR2 KO, and TLR4 KO mice. Multiple studies of murine chlamydial infection report a preponderance of IgG2a vs IgG1, reflective of a Th1-dominant response (1, 34, 35, 37). We found a preponderance of IgG2a in serum taken on day 35 of infection in control, TLR2 KO, and TLR4 KO mice. IgG1 was undetectable in many of the mice in all three groups. Titers of IgG2a were not different among the groups (mean ± SEM log_{10} IgG2a for 10 mice in each group = 2.1 ± 0.2 for C57BL/10 mice, 2.7 ± 0.3 for TLR2 heterozygous littermates, 2.7 ± 0.3 for TLR2 KO mice, and 2.8 ± 0.4 for TLR4 KO mice).

Discussion

Resolution of a primary chlamydial genital tract infection in the mouse ultimately relies on a Th1 cell-mediated immune response (1–4, 38–40). This adaptive immunity in turn is initiated by the innate immune system. It is likely that C. trachomatis initially invades genital tract epithelial cells as well as resident tissue macrophages and dendritic cells. Infected cells respond by releasing inflammatory mediators and chemokines that induce an influx of NK cells and neutrophils into the area, activate nearby phagocytes and APCs, and ultimately dictate the ensuing acquired response. We demonstrated the presence of both TLR2 and TLR4 in the upper and lower genital tract tissues of noninfected and infected mice in our initial experiments, which allowed us to proceed with an examination of their relative importance, if any, in chlamydial infection. Evidence has previously been presented showing that TLRs are key in translating microbial recognition into expression of specific subsets of chemokines and cytokines from macrophages and dendritic cells (13, 21, 29, 41, 42). Furthermore, TLR2 and TLR4 have been shown to induce different sets of chemokines and cytokines, implying that the innate immune response can be customized for different pathogens (43–46). It is therefore vital to elucidate the role of these two receptors in the expression of inflammatory mediators by infected cells in response to C. trachomatis.

The results of the in vitro experiments demonstrated an important contribution of TLR2 in macrophage production of TNF-α and IL-6. In the absence of this receptor, peritoneal macrophages showed a statistically significant reduction in expression of these cytokines. However, despite significant decreases, inhibition was not complete. This finding underscores an important redundancy in the TLR system whereby inflammatory mediators can still be produced, albeit in smaller amounts, by cells lacking only one of these two receptors.

As regards chlamydial infection, infected epithelial cells have been shown to be intimately involved in the early innate response to infection (5, 6, 47). We investigated the role of TLR2 and TLR4 in Chlamydia-induced inflammatory cytokine gene transcription in primary murine fibroblasts and found a significant dependence on TLR2 for production of IL-1, MIP-2, and IL-6 in response to infection with MoPn. However, as with the phagocytic cell response, fibroblasts did not demonstrate complete dependency on TLR2 for activation of a cytokine response upon chlamydial infection.
Other groups have found a similar TLR2 dependency in dendritic cell, peripheral blood mononuclear cell, and macrophage activation after infection in vitro with *C. pneumoniae* (28, 29). No particular chlamydial protein has been definitively proved as a TLR2 ligand, but there are several candidates. Aside from various lipoproteins, chlamydiae may produce a glycansless cell wall polypeptide similar to the known TLR2 ligand PGN (48). Furthermore, some studies have shown that chlamydial heat shock protein 60 (cHSP60) is capable of binding TLR2 and TLR4. There is, however, disagreement in the literature as to which of these two receptors plays a more important role in binding cHSP60, depending on the cell line examined (25, 49–51). Finally, it is generally agreed that endogenous HSPs may bind cells via TLR2 and TLR4, revealing an interesting possibility that TLRs on a cell may be activated by ligands released at the death of an infected cell (26, 50, 52, 53). In this way, the infection could produce induction of inflammatory mediators in an indirect manner, via release of danger signals from the cytosol of the infected host cell (54, 55).

To our knowledge, this body of work is the first to demonstrate activation of TLR2 by *C. trachomatis* as a whole organism, in contrast to other studies that have examined individual components of this pathogen. More importantly, we have shown that only live, actively replicating chlamydiae are capable of inducing an inflammatory response from macrophages, because UV-inactivated EBs were ineffective in inducing cytokine release. Therefore, it can be inferred that during the intracellular transformation of an EB to a reticulate body, or during replication of reticulate bodies, a crucial ligand is presented or produced that can activate TLR2. This is plausible in that a previous study has demonstrated that TLRs are able to sample pathogens that are present in macrophage

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**FIGURE 8.** Intensity and duration of in vivo genital tract infection are not affected by TLR2 or TLR4 deficiency. Quantitative isolations from lower genital tract swabs obtained after infection with MoPn *C. trachomatis* in TLR2+/– and TLR2−/− mice (A) and in TLR4+/– and TLR4−/− mice (B). Data points represent means ± SEM of duplicate determinations with 10 animals examined on each day.

**FIGURE 9.** Oviduct pathology is significantly decreased in TLR2 KO mice. Histopathological evaluation of oviducts from TLR2+/– and TLR2−/− mice on day 35 of infection reveals minimal oviduct dilatation in the TLR2−/− mice (A, ×4; B, ×10; C, ×20), but marked dilatation consistent with hydrosalpinx in the wild-type mice (D, ×4; E, ×10; F, ×20). Arrows, Oviducts.
phagosomes (23). Although activation of TLRs by MoPn at the cell surface cannot be ruled out, the requirement for replication certainly suggests an intracellular localization of ligand binding.

It is interesting that chlamydial LPS associated with the UV-inactivated organism induced minimal cytokine release from macrophages compared with LPS from _E. coli_ that induced TNF and IL-6 in large quantities. However, purified chlamydial LPS is 100-fold less potent than LPS from _Salmonella minnesota_ R595 or the lipo-oligosaccharide from _Neisseria gonorrhoeae_ at inducing an inflammatory cytokine response (56). We have observed by real time PCR >50,000-fold increase in mRNA for TNF in wild-type macrophages infected with live MoPn compared with a 300-fold increase in macrophages exposed to UV-inactivated EBs (our unpublished data). Thus, the decrease is seen at the level of gene transcription as well as protein production.

Of interest are the increased cytokine responses observed at all inoculum levels in TLR4 KO macrophages and fibroblasts. That the TLR4 KO cells responded to MoPn infection by producing more cytokines than controls under the same conditions is significant because it is counterintuitive. One possibility for this discrepancy is that TLR4 KO macrophages have increased expression of other receptors, such as TLR2, and these account for the increased production of cytokines in response to infection. Another possible explanation is that in TLR4 KO cells, heterotolerance effects on NF-κB signaling are removed (57). Heterotolerance to TLR2 ligands induced by TLR4 signaling would not be possible in TLR4-deficient cells, leaving responses mediated via binding to TLR2 uninhibited and accentuated. Thus, heightened TLR2-mediated signaling would overshadow any contribution of TLR4 to macrophage or fibroblast activation. Recently, it was shown that IL-4 down-regulates TLR2 expression on monocytes and dendritic cells (58). We measured IL-4 in supernatants from MoPn-infected wild-type and TLR4 KO macrophages by ELISA and found barely detectable levels of IL-4 (16 ± 4 pg/ml in wild-type and 14 ± 2 in TLR4-deficient macrophages). Thus, TLR4-induced production of IL-4 is not the mechanism for decreased cytokine release observed in wild-type cells compared with TLR4-deficient cells stimulated with _E. coli_ LPS or infected with _C. trachomatis_. The absence of a heightened cytokine response in vivo in TLR4 KO mice could be explained by the increased availability of down-regulatory responses in an in vivo infection model (59–62).

Our in vivo experiments in TLR2 KO mice also yielded unexpected results. Although significantly lower levels of the inflammatory cytokines TNF-α and MIP-2 were found in genital tract secretions of the TLR2-deficient mice than in controls during the first 2 weeks of infection, there was no difference in the course of infection between the two groups. The cytokine response that remained in the absence of TLR2 signaling was sufficient to recruit effector cells to the genital tract, resulting in equivalent eradication of organisms even in the early days after infection. The similar time to clearance of organisms from the lower genital tract also speaks to the redundancy of the innate immune system; even in the absence of TLR2, the infection is cleared at a normal rate, indicating that other mechanisms can still initiate an effective acquired immune response.

The lack of a significant reduction in IFN-γ and IL-6 in TLR2 KO mice may explain their ability to effectively eradicate genital tract infection with _C. trachomatis_. IFN-γ is clearly important in host defense against chlamydiae, with direct and indirect inhibitory effects demonstrated in vitro (63–69) and in vivo (2–4, 37, 70–72). IL-6 has been shown to be partly responsible for blocking the suppressor activity of CD4+CD25+regulatory T cells (T<sub>R</sub>), allowing for activation of pathogen-specific adaptive immune responses (73). The efficient resolution of genital tract infection observed in the TLR2 KO mice, implies that an intact adaptive CD4<sup>+</sup>Th1 response occurred in these mice, given that these cells are essential for eradication of genital infection in the murine model (38, 74). The detection of normal titers of IgG2a Abs in the TLR2 KO mice also suggests that a normal Th1 response occurred in these mice, resulting in an infection course resembling that in mice with intact TLR2 function.

Histopathology data revealed that the presence of TLR2 contributes to chronic inflammatory sequelae found after infection has resolved. It is possible that the TLR2 KO mice cleared upper tract infection faster than control mice, resulting in a more rapid resolution of inflammation and subsequent decrease in chronic oviduct pathology. However, the absolutely comparable rate of resolution of genital tract infection between the two groups. The cytokine response that remained in the absence of TLR2 signaling was sufficient to recruits effector cells to the genital tract, resulting in equivalent eradication of organisms even in the early days after infection. The similar time to clearance of organisms from the lower genital tract also speaks to the redundancy of the innate immune system; even in the absence of TLR2, the infection is cleared at a normal rate, indicating that other mechanisms can still initiate an effective acquired immune response.

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![Figure 10](http://www.immune.org/)
TLR2 IN CHLAMYDIAL GENITAL INFECTION

TLR4, the intact organism stimulates innate immune cells and the interaction of Chlamydia trachomatis Igs-positive Dutch women. Thomas Jungas.

We acknowledge the superb technical assistance of Jim D. Sikes and Acknowledgments 6196 TLR2 IN CHLAMYDIAL GENITAL INFECTION.

responses, although significantly, this is in contrast to our model in which cytokine responses observed in the TLR4 KO mice and controls are reflected in similar outcomes of infection. These data argue against a direct role for chsP60-induced TLR4-mediated tissue damage in genital tract infection but does not rule out a role for HSP60 in the pathological process. Interestingly, a recent report by Morre et al. (77) found no association of the functional polymorphism in the gene encoding TLR4 function (Asp299Gly) with tubal infertility in C. trachomatis IgG-positive Dutch women.

In conclusion, this study revealed important information regarding the interaction of C. trachomatis with TLRs. Although parts of this organism (chlamydial LPS and chsP60) are recognized by TLR4, the intact organism stimulates innate immune cells and fibrinolysin independently of TLR4. TLR2 plays a significant role as a pattern recognition receptor for this organism, and stimulation of TLR2 requires active infection with Chlamydia. The normal clearance of organisms from the genital tracts of TLR2 KO mice and the nonsensitization reduction in key cytokines in these mice point to the redundancy of the immune response and the number of collateral pathways available for innate responses to infections. Importantly, the in vivo data indicate a protective role for TLR2 deficiency in genital tract infection sequelae due to C. trachomatis, given that mice deficient in TLR2 exhibited significantly decreased ‘odious pathology. The remarkably improved outcome in these mice suggests that an improved balance in protective vs pathological inflammatory responses occurs with TLR2 deficiency with respect to Chlamydia infection of the female genital tract.

Acknowledgments

We acknowledge the superb technical assistance of Jim D. Sikes and Thomas Jungas.

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