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Pattern Recognition Molecules Activated by *Chlamydia muridarum* Infection of Cloned Murine Oviduct Epithelial Cell Lines[1]

Wilbert A. Derbigny, Micah S. Kerr, and Raymond M. Johnson[2]

***Chlamydia trachomatis* is the most common bacterial sexually transmitted disease in the United States and a major cause of female infertility due to infection-induced Fallopian tube scarring. Epithelial cells are likely central to host defense and pathophysiology as they are the principal cell type productively infected by *C. trachomatis*. We generated cloned murine oviduct epithelial cell lines without viral or chemical transformation to investigate the role of the TLRs and cytosolic nucleotide binding site/leucine-rich repeat proteins Nod1 and Nod2 in epithelial responses to *Chlamydia muridarum* infection. RT-PCR assays detected mRNA for TLR2 (TLRs 1 and 6), TLR3, and TLR5. No mRNA was detected for TLRs 4, 7, 8, and 9. Messenger RNAs for Nod1 and Nod2 were present in the epithelial cell lines. Oviduct epithelial cell lines infected with *C. muridarum* or exposed to the TLR2 agonist peptidoglycan secreted representative acute phase cytokines IL-6 and GM-CSF in a MyD88-dependent fashion. Infected epithelial cell lines secreted the immunomodulatory cytokine IFN-β, even though *C. muridarum* does not have a clear pathogen-associated molecular pattern (PAMP) for triggering IFN-β transcription. The oviduct epithelial lines did not secrete IFN-β in response to the TLR2 agonist peptidoglycan or to the TLR3 agonist poly(I:C). Our data identify TLR2 as the principal TLR responsible for secretion of acute phase cytokines by *C. muridarum*-infected oviduct epithelial cell lines. The pattern recognition molecule responsible for infection-induced IFN-β secretion by oviduct epithelial cells remains to be determined.** ***The Journal of Immunology*, 2005, 175: 6065–6075.**

Urogenital serovars of *Chlamydia trachomatis* cause ~3 million sexually transmitted genital tract infections in the United States each year (1), with similar rates of infection in European countries (Ref 2, ⟨www.eurosurveillance.org/ew/2004/041007.asp#5.⟩). Infections by the urogenital *Chlamydia* serovars cause urethritis, cervicitis, epididymitis, pelvic inflammatory disease, neonatal conjunctivitis, and neonatal pneumonia. In women, progression of *C. trachomatis* infection into the upper reproductive tract causes significant inflammation and injury to the Fallopian tubes (3). *Chlamydia*-induced scarring of the Fallopian tubes is the cause of ~50% of tubal factor infertility and 25% of ectopic pregnancies in developed countries (4, 5).

The pathophysiology underlying *Chlamydia*-induced Fallopian tube scarring is unclear. In mice, experimental *Chlamydia muridarum* genital tract infections are spontaneously cleared by T lymphocytes in a Th1-dependent fashion (6). In humans, the adaptive immune response to *C. trachomatis* infection is also Th1 mediated (7). It has generally been accepted that immunopathologic T cell responses to the infection cause Fallopian tube scarring (8). However, recent data in mice (9) and humans (10–13) reveal that *Chlamydia* infections of the genital tract can persist for long periods of time. Because *Chlamydia*-infected epithelial cells secrete proinflammatory cytokines (14–19), it is possible that infected epithelial cells play a dominant role in the pathophysiology of scarring. The hypothesis that scarring of Fallopian tubes is driven by infected epithelial cells, rather than T lymphocytes responding to the infection, has been referred to as the cellular paradigm of *Chlamydia* pathophysiology (20). Consistent with that paradigm, we recently reported that a cloned oviduct epithelial cell line responded to infection by *C. muridarum* with a plethora of cytokines (14), including acute phase cytokines IL-1, IL-6, TNF-α, GM-CSF, and TGF-α. These cytokines have been shown to contribute to the pathophysiology of scarring and fibrosis in other systems (21–27).

Mammalian cells sense the presence of invading microbial pathogens through recognition of pathogen-associated molecular patterns (PAMPs)[3] present in microbial structural subunits including microbial cell wall (e.g., peptidoglycan), cell membrane (e.g., LPS), and virulence proteins (e.g., flagellin) (28). TLRs are membrane-bound receptors that bind PAMPs and trigger host defense responses, including cytokine secretion. At least 11 TLRs have been identified in mammals. Humans have TLR1–10; mice have TLR1–9 and 11 (28, 29). In addition to TLRs, mammalian cells have cytosolic surveillance for PAMPs via the nucleotide binding site/leucine-rich repeat (NBS/LRR) proteins, Nod1 and Nod2.

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[3] Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; NBS/LRR, nucleotide binding site/leucine-rich repeat; PRM, pattern recognition molecule; ODN, oligonucleotide; DN, dominant negative; IFU, inclusion-forming unit; si, small interfering; hfh-4, helix factor hepatocyte NF/forkhead homologue-4; mogp-1, mammalian oviduct-specific glycoprotein; PGN-EC, *E. coli* peptidoglycan; siSCR, scrambled RNA sequence siRNA; IRF, IFN regulatory factor; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-β; TICAM, Toll-IL-1R domain-containing adaptor molecule.

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Nod1 is activated by a peptidoglycan motif found in Gram-negative bacteria (30, 31), while Nod2 binds muramyl dipeptides (32, 33).

Previous work in humans has shown that primary and immortalized epithelial cells derived from the ectocervix and endocervix express TLRs 1–6, but lack TLR4 (34), while a transformed human uterine epithelial cell line (35) and primary uterine epithelial cells (36) express TLRs 1–9, including TLR4. RT-PCR and Western blot analysis done on homogenates of Fallopian tube tissue show the presence of TLR2 and TLR4 in the upper reproductive tract (37). Similarly, homogenates of murine oviducts have detectable TLR4 mRNA by RT-PCR (38). However, analysis of TLR expression in Fallopian tube and oviduct homogenates includes all cell lineages (epithelium, endothelium, fibroblasts, myeloid, and lymphoid) resident within the organ. To date, the TLR repertoire expressed on upper reproductive tract epithelial cells has not been determined.

Activation of upper reproductive tract epithelial cells via pattern recognition molecules (PRMs) likely plays an important role in the pathophysiology caused by *Chlamydia* infection. The PRMs used by reproductive tract epithelium to "sense" and respond to invasion by *C. muridarum* are not known. However, previous work using TLR2- and TLR4-knockout mice has identified TLR2 as a major contributor to infection-induced cytokine secretion and oviduct pathology, without contributing to the clearance of *Chlamydia* infection from the genital tract (38). In this report, we investigate the PRMs expressed by murine oviduct epithelial cell lines and their roles in *C. muridarum*-induced secretion of epithelial cytokines.

Materials and Methods

Reagents

The following TLR agonists were purchased from InvivoGen: 1) ultrapure LPS of *Escherichia coli* serotype 0111:B4 (1×10^6 endotoxin units (EU)/mg); 2) Peptidoglycan from *E. coli* serotype 0111:B4 (125 EU/mg); 3) the synthetic tripalmitoylated lipoprotein analog Pam_3CSK4 (<0.125 EU/mg); 4) flagellin purified from *Salmonella typhimurium* (125 EU/mg); 5) ODN1826, an oligonucleotide (ODN) containing murine CpGs (<0.125 EU/mg); and 6) ODN1826 control (ODN control), a control ODN without CpGs (<0.125 EU/mg). Poly(I:C), product number P-0913, was purchased from Sigma-Aldrich.

Mice

Female B6.C-H2^{bm12}/KhEg and B6.C-H2^{bm1}/ByJ mice were purchased from The Jackson Laboratory and housed in Indiana University-Purdue University specific pathogen-free facilities. The Institutional Animal Care and Utilization Committee approved all experimental protocols.

Cells, plasmids, and bacteria

RAW264.7 cells (American Type Culture Collection) were cultured at 37°C in a 5% CO_2 humidified incubator and maintained in DMEM supplemented with 10% (v/v) Fetalclone III (HyClone). The cloned oviduct epithelial cell line Bm1.11 has been described previously (14), and a new cloned epithelial cell line described in this report, designated Bm12.4, was derived using a similar methodology. Reproductive tract tissues from a B6.C-H2^{bm12}/KhEg female mouse encompassing the proximal half of the uterine horn to the oviduct tissue immediately adjacent the ovary were harvested. Lumenal epithelial cells were released with PHC mixture as previously described for the Bm1.11 cell line (14). The resulting epithelial cell-enriched cells were expanded in vitro and cloned by limiting dilution after passage through a 0.40-μM filter to remove cell clumps. Resulting clones were screened for IFN-γ-inducible MHC class II (feature of murine epithelial cells but not generally murine fibroblasts). A selected clone with inducible MHC class II expression and epithelial morphology was limiting-diluted a second time to ensure clonality and the resulting clone designated Bm12.4. The epithelial lineage of Bm12.4 was confirmed by immunohistochemical staining for cytokeratin intermediate filaments. The upper reproductive tract epithelial cells were grown at 37°C in a 5% CO_2 humidified incubator and maintained in epithelial-cell medium: 1:1 DMEM:F12K (Sigma-Aldrich), supplemented with 10% characterized FBS (HyClone), 2 mM L-alanyl-L-glutamine (Glutamax I; Invitrogen Life Technologies), 5 μg of bovine insulin/ml, and 12.5 ng/ml recombinant human fibroblast growth factor-7 (keratinocyte growth factor; Sigma-Aldrich).

The dominant-negative (DN) MyD88 expression plasmid pIRES2-EGFP-DN-MyD88 (39) was generously provided by Dr. S.-C. Hong (Indiana University School of Medicine, Indianapolis, IN).

Mycoplasma-free *C. muridarum*, previously known as *C. trachomatis* strain MoPn, was grown in McCoy cells (American Type Culture Collection). The titers of *Mycoplasma*-free *C. muridarum* stocks were determined on McCoy cells with centrifugation as described previously (14, 40).

Immunohistochemistry and flow cytometry

Bm12.4 cells grown on glass chamber slides (Labtek) were fixed for 2 min with ice-cold methanol, blocked with 5% normal goat serum/PBS for 45 min, then stained with mAb mixture AE1/AE3 specific for acidic and basic cytokeratins (Cappel/ICN) or 36-7-5, a control mAb specific for H-2K^k (epitope not present on Bm12.4 cells) (BD Pharmingen). Detection was accomplished with a FITC-coupled goat anti-mouse IgG F(ab′)$_2$ affinity-purified antiserum (Cappel/ICN).

For flow cytometry, Bm12.4 cells were dislodged from tissue culture plastic using a Hank's salt-based, enzyme-free cell dissociation buffer (Sigma-Aldrich). Cells were stained for 20 min on ice in PBS/2%BSA with PE-coupled M5/114.15.2 (I-A^b) (eBioscience). IFN-γ induction was accomplished by addition of 10 ηg/ml murine rIFN-γ (Sigma-Aldrich) to the culture medium for 14 h before staining. Cells were analyzed with a FACSCalibur cytometer (BD Biosciences).

Infections

Bm1.11, Bm12.4, and RAW 264.7 cells were plated in 48-well tissue culture plates and used when confluent. For all experiments, the cells were infected with 10 inclusion-forming-units (IFU) of *C. muridarum*/cell in 900 μl of culture medium. The 48-well plates were centrifuged at 1000 rpm ($300 \times g$) in a table-top centrifuge for 30 min then incubated at 37°C in a 5% CO_2 humidified incubator without subsequent change of medium for 24–30 h, depending upon the assay. Mock-infected wells received an equivalent volume of sucrose-phosphate-glutamic acid buffer lacking *C. muridarum*.

Transfection

To generate the DN MyD88-expressing cell line designated DN-MyD88, Bm1.11 cells were transfected with 5 μg of the pIRES2-EGFP-DN-MyD88 plasmid in 75% confluent 6-well plates using LipofectAMINE 2000 reagent (Invitrogen Life Technologies). After 48 h, the cells were removed from the plate using enzyme-free cell dissociation buffer (Sigma-Aldrich) and passed through 40-μM nylon cell mesh to remove clumps of cells. The cells were suspended in cell-sorting buffer (PBS + 2% FCS) for screening GFP expression (incorporated in the plasmid), using the FACSVantage SE cell sorter (BD Bioscience). Clones were obtained by limiting dilution in epithelial-cell medium supplemented with 800 μg/ml G418. Selected clones were screened for expression of the DN MyD88 protein by Western blot analyses. The DN-MyD88 cell line was maintained in epithelial-cell medium supplemented with 400 μg/ml G418.

17-β-estradiol treatment of Bm1.11 and Bm12.4 cells

Early passage (<32) Bm1.11 and Bm12.4 cells were set up in RPMI 1640 medium lacking phenol red and supplemented with 10% characterized FBS (HyClone), 2 mM L-alanyl-L-glutamine (Glutamax I; Invitrogen Life Technologies), 5 μg of bovine insulin/ml, 12.5 ηg/ml recombinant human fibroblast growth factor-7 (keratinocyte growth factor; Sigma-Aldrich) and 10^{-8} M 17-β-estradiol (Sigma-Aldrich) for 48 h before isolating total RNA for RT-PCR analysis.

Western blotting

Control and DN-MyD88 Bm1.11 cells were grown in monolayers in a 6-well plate to confluence. After removal of the growth medium, the monolayers were gently washed with PBS, and cytosolic proteins were recovered in the cell fractionation buffer provided in the PARIS kit (Ambion). Cytosolic proteins were quantified using the Micro BCA Protein Assay kit (Pierce). Twenty-five micrograms of lysate from either the DN-MyD88 or nontransfected Bm1.11 cell line were boiled in 5× Immunopure Reducing Sample Buffer (Pierce) before SDS PAGE. After separation by 10% SDS-PAGE, the proteins were transferred to Immobilon-P (Millipore) transfer membranes. Transfer membranes were blocked in 5% nonfat dry milk and subsequent immunoblotting was performed using a 1/5000 dilution of the MyD88-specific polyclonal Ab HFL-296 (Santa Cruz Biotechnology) in TBST buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween

20). The primary Ab incubation, performed for 1 h at room temperature, was followed by three TBST washes. Membranes were then incubated with 1/10,000 HRP-conjugated goat anti-rabbit polyclonal Ab (Amersham Biosciences) for 1 h, followed by three washes in TBST. Proteins were visualized via chemiluminescence using the ECL plus Western blotting detection system (Amersham Biosciences) as described in the manufacturer's protocol.

RNA interference

MyD88-specific small interfering (si)RNA, MyD88-specific primers, scrambled control siRNA, siRNA transfection reagent, and siRNA transfection medium were purchased from Santa Cruz Biotechnology. siRNA transfections targeting endogenous MyD88 and scrambled controls were conducted in 48-well plates seeded with 4×10^4 Bm1.11 cells/well using the manufacturer's protocol. The siRNA mixture was mixed with the transfection reagent mix and allowed to form liposome-siRNA complexes for 30 min. After initial 5 h of incubation, the transfection medium was replaced with fresh epithelial medium, and the cells were returned to the 37°C CO_2 humidified incubator for 30 h before using them in experiments.

Real-time PCR

Cytoplasmic RNA was purified from the siRNA transfected Bm1.11 cells using the PARIS kit (Ambion). The RNA was quantified by spectrophotometric analysis, and RNA integrity was confirmed by agarose gel electrophoresis. cDNA synthesis was performed using 1 μg of the cytoplasmic RNA with the iScript cDNA synthesis kit (Bio-Rad). The cDNA product was diluted 1/50, while the MyD88-specific and β-actin control primers (Table I) were adjusted to 10 pmol/μl working stock. Real-time PCR was conducted with the diluted cDNA and primers as per the protocol outlined in the iTaq SYBR Green Supermix with ROX kit (Bio-Rad). Real-time PCR was performed with an ABI Prism 7700 machine (Applied Biosystems): 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 2 min at 60°C. Cycle threshold (C_T) values were determined by automated threshold analysis with ABI Prism version 1.0 software. The amplification efficiencies were determined by serial dilution and calculated as $E = \exp^{-1/m}$, where E is the amplification efficiency and m is the slope of the dilution curve (41). Dissociation curves were recorded after each run to ensure primer specificity.

RT-PCR

Total RNA was isolated from oviduct epithelial cell lines using RNeasy mini columns (Qiagen). Total RNA enriched in luminal epithelial cell RNA was isolated from the upper reproductive tract and terminal ileum of a female B6.C-H2^{bm1}/ByJ mouse. A segment of the upper reproductive tract extending from the mid-uterine horn to immediately adjacent to the ovary was resected, as well as a segment of terminal ileum of equivalent length. The lumen of the terminal ileum was cannulated and gently flushed with 2 cc of PBS. Total RNA was extracted from the lumens of the upper reproductive tract and terminal ileum by cannulating each with a 28-gauge needle attached to a 0.5-cc syringe and running 0.5 ml of cell lysing solution containing guanidine thiocyanate (Qiagen RLT buffer) through the lumen of each organ to preferentially lyse the luminal epithelial cells while preserving the organs general architecture. Total RNA within the luminal flow-through was isolated using RNeasy mini columns (Qiagen). During purification, all RNA samples were treated with RNase-free DNase I (Qiagen) to remove genomic DNA contamination. The RNA was quantified by spectrophotometric analysis. For TLR analyses, RNA integrity was confirmed by agarose gel electrophoresis. Optimized primer pairs were designed using Vector NTI Suite (Infomax). Specific primer pairs (Amitof) are listed in Table I. Using 1 μg of total RNA as the template for each reaction, RT-PCR was accomplished by using a single-tube avian myeloblastosis virus RT-Tfl polymerase kit (Access RT-PCR; Promega). Cycling conditions were as follows: 90 s of initial denaturation at 95°C, followed by eight cycles, each of which consisted of 30 s at 95°C, 15 s at 56°C, and 30 s at 72°C. After the initial eight cycles, the PCR continued for 32 additional cycles of 30 s at 95°C, 15 s at 56°C, and 30 s + 3 s/cycle at 72°C, followed by a final extension at 72°C for 3 min. Reactions were also amplified in the absence of reverse transcriptase as negative controls. The semiquantitative RT-PCR technique used to generate qualitative data from the siRNA experiments used a two-step PCR event using a proprietary nested-primer set for MyD88 and the β-actin control per the manufacturer's protocol (Santa Cruz Biotechnology).

ELISA determination of cytokine production

RAW264.7 cells and confluent Bm1.11 and Bm12.4 monolayers grown in 48-well tissue culture-treated plates were either infected with 10 IFU of *C. muridarum*/cell or treated with the appropriate TLR ligand at the concentrations specified in the text. Supernatants were harvested at either the 24- or 36-h time points and analyzed for cytokine content using ELISAs for IL-6 and GM-CSF according to the manufacturer's protocol. The anti-mouse IL-6 mAb 32C11, biotin-labeled anti-mouse IL-6 Ab 20F3, recombinant murine IL-6, and recombinant murine GM-CSF were purchased from Endogen. The anti-mouse GM-CSF mAb MP1-22E9, biotin-labeled anti-mouse GM-CSF mAb MP1-31G6, and the streptavidin-HRP conjugate were purchased from BD Pharmingen. For the IFN-β ELISA, the rabbit polyclonal anti-IFN-β capture Ab (R&D Systems) was used at a 1/250 dilution in bicarbonate buffer; the anti-IFN-β detection rat monoclonal 8.S.415 (U.S. Biological) was used at 1 μg/ml; the rabbit anti-rat HRP conjugate (Jackson ImmunoResearch Laboratories) was used at 1/10,000. All standards and experimental samples were done in triplicate. The lower range of assay sensitivity for individual cytokines was 50 pg/ml for IL-6 and 10 pg/ml for GM-CSF and IFN-β. All experiments were repeated at least three times, and significance was determined using Student's t test.

Results

Cloned murine oviduct epithelial cell lines

We previously published the derivation of a cloned oviduct epithelial cell line without viral or chemical transformation, designated Bm1.11, from a B6.C-H2^{bm1}/ByJ mouse (14). We have derived a second cloned oviduct epithelial cell line without viral or chemical transformation, designated Bm12.4, from a B6.C-H2^{bm12}/KhEg mouse using a similar methodology. Characteristic

Table I. *Primers used for RT-PCR*

Product	Sense Primer	Antisense Primer	PCR Product Size (bp)
TLR1	5′-GTGAATGCAGTTGGTGAAGAAC-3′	5′-GCTCATTGTGGGACAAATCCAA-3′	450
TLR2	5′-CTTGTTTCTGAGTGTAGGGGCT-3′	5′-CGAACCAGGAGGAAGATAAACT-3′	483
TLR3	5′-ACCCTTTCAAAAACCAGAAGAATC-3′	5′-GGACAGACGCTGTATATTGTTG-3′	521
TLR4	5′-TCAACCCCTTGAAGATCTTAAA-3′	5′-CAATTGGGTTCAAAGACATGTC-3′	459
TLR5	5′-CAGTATCAGCTGATGAGACATGAG-3′	5′-GACAGTACCGCAATAGGGATGG-3′	463
TLR6	5′-TACGGAGCCTTGATTTCCATGT-3′	5′-TGGACCTCTGGTGAGTTCTGAT-3′	485
TLR7	5′-AACCACATACCAAGCATCTCTC-3′	5′-AAATTAGGTGGCAAAGTGGTGG-3′	458
TLR8	5′-CAGAGTTGGATGTTAAGAGAGA-3′	5′-GTATATAACTGGTTGTCTTCCA-3′	459
TLR9	5′-GCCTGAGCCACACCAACATCCT-3′	5′-CCAGACCTTGGAACCAGGAAGA-3′	477
Nod1	5′-TCACCCTCTGGTCCTGCTGGCTAA-3′	5′-TCCTCCTGGCCAAACACAAAGA-3′	525
Nod2	5′-GAGGAGCTTCCAGGAGTTTCTC-3′	5′-AAGACAGGGAGGTGGCACAAAC-3′	507
MyD88	5′-ATCCGGGTCCCTGGACTCCTTCAT-3′	5′-TGGTGATGCCTCCCAGTTCCTT-3′	488
Hfh-4	5′-CCTGACGACGTGGACTATGCCA-3′	5′-TGTGGCCTCCTCAAACTCCTGA-3′	450
Mogp-1	5′-TGTCGGCTGCTGTCTCTGGCAT-3′	5′-CCCCTGACATCGTCCATATCCA-3′	550
PigR	5′-AACCGAGGCCTGTCCTTCGATG-3′	5′-CCTCAGGCCTGTGATCAACACA-3′	600
β-actin	5′-ATGGATGACGATATCGCTGAGC-3′	5′-CGTACATGGCTGGGGTGTTGAA′	400

of an epithelial lineage, Bm12.4 cells express cytokeratins (42) and have IFN-γ-inducible MHC class II expression (Fig. 1).

To confirm their anatomical site of origin, RT-PCR analysis for oviduct-specific mRNAs helix factor hepatocyte NF/forkhead homologue-4 (hfh-4) and mammalian oviduct-specific glycoprotein (mogp-1) were performed on Bm1.11 and Bm12.4 cell lines ~30 passages after clonal derivation. Hfh-4 and mogp-1 are expressed by oviduct but not uterine epithelium (43). hfh-4 is expressed by ciliated oviduct epithelial cells (44), while mogp-1 is expressed by secretory oviduct epithelial cells (45). The expression of hfh-4 and mopg-1 is mutually exclusive within individual cells. No mRNA could be detected for mogp-1 in either cell line under optimal conditions for its expression (10^{-8} M 17-β-estradiol in phenol red-free medium), while hfh-4 mRNA was detectable in Bm1.11 and Bm12.4 cells (Fig. 2). Detection of hfh-4 mRNA but not mogp-1 mRNA is consistent with Bm1.11 and Bm12.4 cells being of ciliated oviduct epithelial origin. Low levels of hfh-4 mRNA are not anomalous as cultured oviduct ciliated epithelial cells dedifferentiate and rapidly loose their cilia ex vivo (46). Absence of mRNA for the polymeric Ig receptor indicates that ex vivo culturing of these cell lines under the conditions used has altered some aspects of their biology compared with oviduct epithelial cells in vivo.

Identification of the PRMs expressed by oviduct epithelial cells

The mock-infected and *C. muridarum*-infected (10 IFU/cell) mouse oviduct epithelial cell lines Bm1.11 and Bm12.4 were examined by RT-PCR for expression of TLRs 1–9 and the NBS/LLR proteins Nod1 and Nod2. Recently discovered murine TLR11 (29) was not investigated, and human TLR10 does not have a murine homologue. For expression analysis, 1 μg of total cellular RNA was subjected to a 40-cycle RT-PCR using the primers listed in Table I. All primer pairs were shown to generate specific PCR products using total RNA from peritoneal macrophages or the RAW264.7 macrophage cell line (data not shown). As shown in Fig. 3*A*, both uninfected cell lines had detectable mRNA for TLRs 1, 2, 3, 5, and 6 and Nod1. The faint PCR products for TLRs 1, 3, and 5 in Bm12.4 cells were readily demonstrable by increasing the cycle number to 48 (Fig. 3*B*). There were qualitative differences in the mRNA expression levels for TLRs 1, 3, and 5 between the Bm1.11 and Bm12.4 cell lines. Expression of TLR4 mRNA was undetectable in both cell lines even with a 48-cycle RT-PCR.

To examine whether infection with *Chlamydia* alters PRM expression, both cell lines were infected with *C. muridarum* at 10

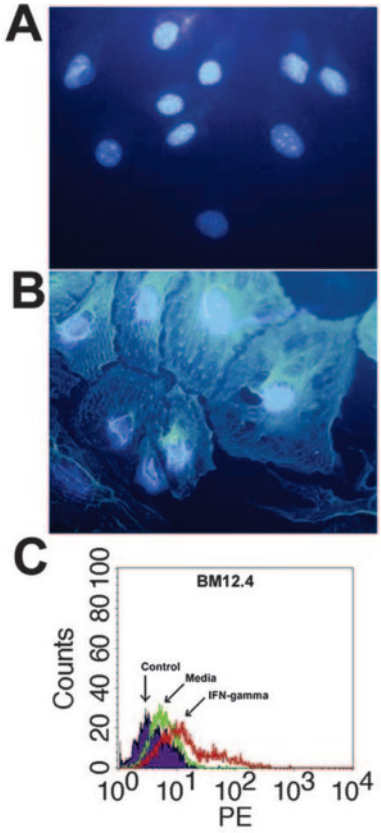

FIGURE 1. Oviduct epithelial cell line Bm12.4. *A*, Control staining with anti-H-2K^k Ab. Nuclei counterstained with 4′,6′-diamidino-2-phenylindole. *B*, Staining with anti-cytokeratin Abs. Nuclei counterstained with 4′,6′-diamidino-2-phenylindole. *C*, Flow cytometry showing expression of MHC class II I-A^{bm12} at baseline and after 14 h exposure to IFN-γ (10 ηg/ml).

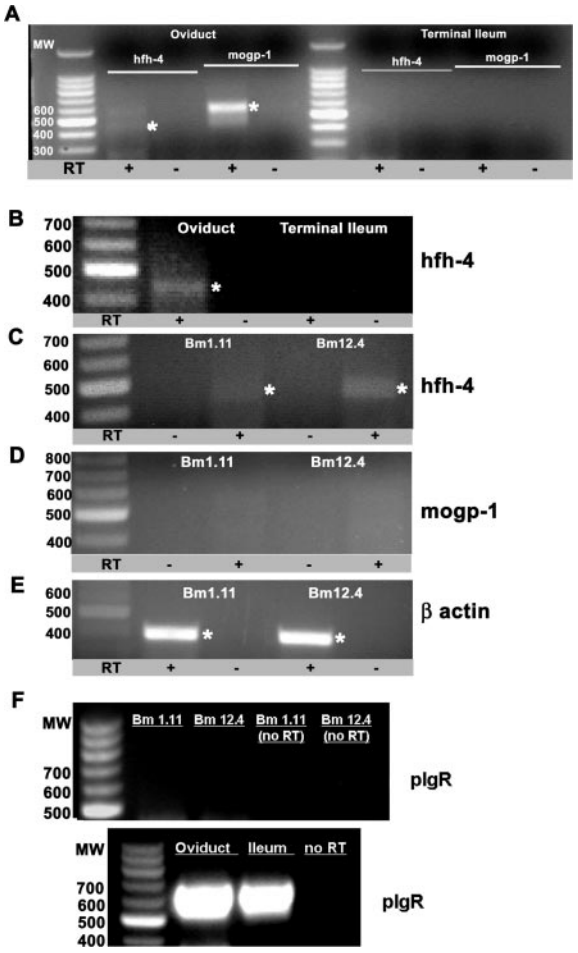

FIGURE 2. Lineage characterization of Bm1.11 and Bm12.4 epithelial cell lines. *A*, Forty-cycle RT-PCR using 1 μg of epithelial cell-enriched total RNA from the oviduct and terminal ileum for hfh-4 mRNA (marker for oviduct ciliated epithelium) and mogp-1 (marker for oviduct secretory epithelium). *B*, Forty-eight-cycle RT-PCR for hfh-4 using 1 μg of epithelial-enriched total RNA from the oviduct and terminal ileum. *C*, Forty-eight-cycle RT-PCR, using 2 μg of total RNA, demonstrating the presence of hfh-4 mRNA in Bm1.11 and Bm12.4 cell lines. *D*, Forty-eight-cycle RT-PCR using 2 μg of total RNA demonstrating the absence of mogp-1 in Bm1.11 and Bm12.4 cell lines. *E*, β-Actin control for Bm1.11 and Bm12.4 RNA. *F*, No mRNA for the polymeric IgR was detected in the Bm1.11 and Bm12.4 cell lines. MW, m.w. in base pairs. RT, reverse transcriptase; +, RT present; −. RT absent.

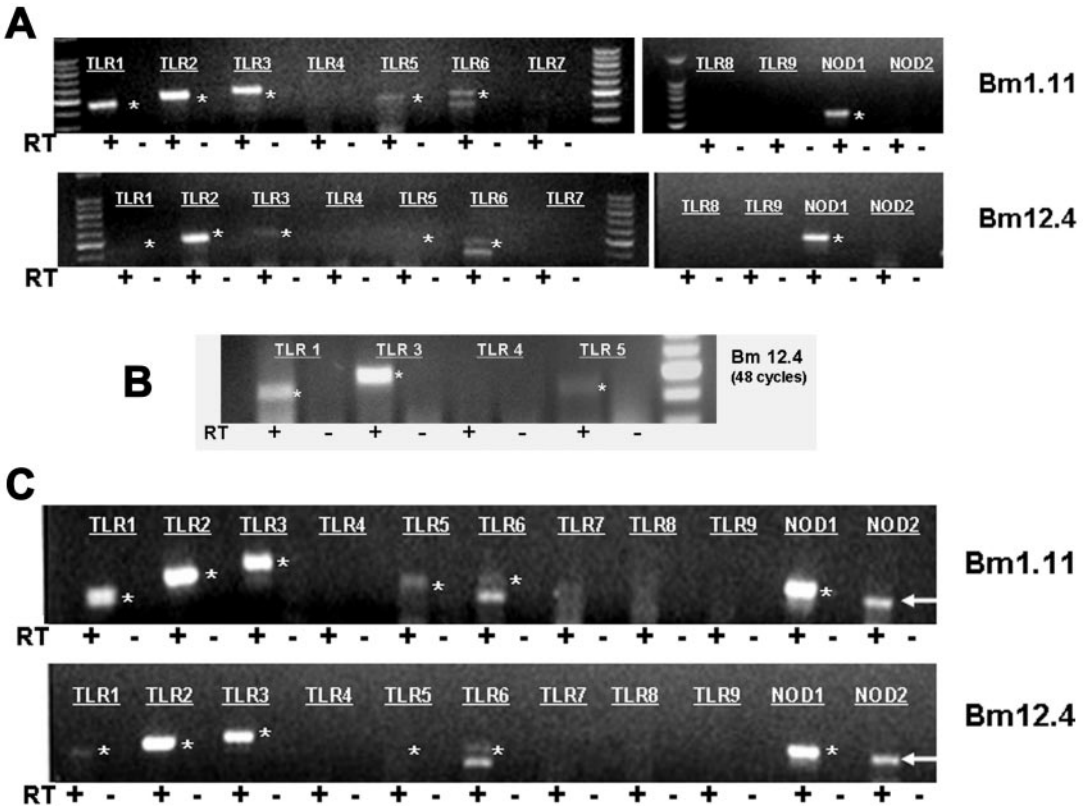

FIGURE 3. TLR expression in oviduct epithelial cells. *A*, Total cell RNA was extracted from Bm1.11 and Bm12.4 cells, reverse transcribed, and amplified with the PRM primers listed in Table I. The RT reactions were performed in both the presence (+) and absence (−) of the AMV RT. *B*, Extending the Bm12.4 RT-PCR from 40 to 48 cycles for TLRs 1, 3, 4, and 5. The faint TLR5 PCR product was gel purified, cloned, and sequenced to confirm its identity. *C*, Bm1.11 and Bm12.4 cells were infected with *C. muridarum* at 10 IFU/cell and total cell RNA harvested at 24 h postinfection for analysis by RT-PCR. Where present, the specific PCR products are indicated by the asterisks. The arrows highlight the induced expression of Nod2 at 24 h postinfection. The band below the TLR6 RT-PCR product is a spurious band resulting from limited primer pair homologies (13 of 22 bp in each primer) with a murine Na^+/K^+ transporter ATPase (BC034177).

IFU/cell, and RT-PCR analysis was performed at 6, 12, 18, and 24 h postinfection. Infection-induced changes in TLR expression were first noted at 18 h postinfection and increased further by 24 h postinfection. The only qualitative changes seen during the above time course were increases in TLR mRNAs. No TLR message significantly decreased or vacillated up and down during the time course examined. Representative data from the 24 h time point are presented in Fig. 3*C*. Interestingly, both cell lines exhibited strong induction of Nod2 24 h postinfection (indicated by the arrow). Nod2 mRNA is known to be up-regulated by TNF-α (47). We have previously shown the TNF-α mRNA is induced in infected Bm1.11 cells and that TNF-α protein is detectable in infected-Bm1.11 culture medium (14). There was no detectable TLR4 mRNA at any time point postinfection (or in uninfected cells), suggesting TLR4 does not play a role in oviduct epithelial activation by *Chlamydia* infection.

Assessment of functional status of TLRs expressed by uninfected oviduct epithelial cells

To assess the functional status of the identified TLRs, uninfected oviduct epithelial cell lines were challenged with highly purified TLR-specific agonists. TLR activation was determined by measurement of epithelial cytokines in cell culture supernatants. IL-6 and GM-CSF were chosen as representative acute phase cytokines and IFN-β as a representative immunomodulatory cytokine, based on our previous work in Bm1.11 cells (14). Even though *Chlamydiae* do not have a TLR5 PAMP (flagellin), we chose to characterize TLR5 because pelvic inflammatory disease often includes bacterial species from the lower reproductive tract. TLR9 was included in the functional analysis because *Chlamydia* DNA contains TLR9 PAMPs, unmethylated cytosines within CpG motifs. Because mRNAs for TLRs 7 and 8 were not detected with RT-PCR analysis, and *Chlamydia* lack their relevant PAMPs (viral ssRNA; Refs. 48 and 49), we chose not to address those PRMs further in this study.

Functional characterization of TLR5 and TLR9 in uninfected Bm1.11 and Bm12.4 cells

TLR5 mRNA was detected in both epithelial cell lines. The PAMP recognized by TLR5 is flagellin, a component of the flagellar motility apparatus used by many pathogenic bacteria. Because *C. trachomatis* and *C. muridarum* genomes do not encode a flagellin (50), TLR5 is not likely to play a role in epithelial activation during uncomplicated *C. trachomatis* genital tract infections but may play a role in polymicrobial pelvic inflammatory disease. Exposure of the oviduct epithelial cell lines to purified *E. coli* flagellin caused a modest dose-dependent secretion of IL-6 and GM-CSF in both cell lines consistent with functional TLR5 molecules (Fig. 4). Low levels of LPS contaminating the purified flagellin (~12.5 pg/ml LPS in 100 ng/ml flagellin) are unlikely to account for the IL-6 production seen in Fig. 4 based on the poor IL-6 response of the oviduct epithelial cell lines to μg/ml quantities of LPS (upcoming section and see Fig. 6). Unmethylated CpG dinucleotide motifs within consensus sequences in bacterial and viral DNA are

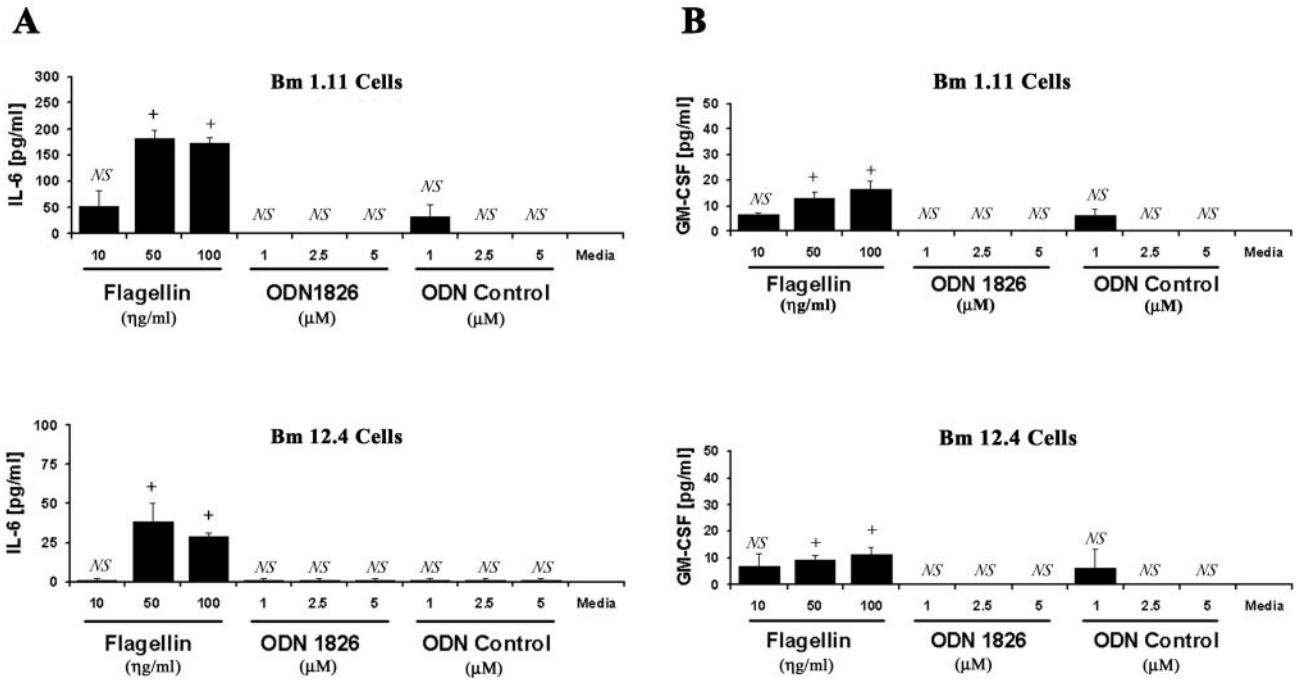

FIGURE 4. Functional analyses of the TLR5 and TLR9 receptors. IL-6 (*A*) and GM-CSF (*B*) levels detected in supernatants from Bm1.11 and Bm12.4 cells stimulated with flagellin and ODN1826, testing for functional TLR5 and TLR9, respectively. The cells were treated with the concentration ranges specified, and supernatants were collected at 30 h for analyses. Data presented are representative of at least three independent experiments. Statistical significance was assigned by comparing the treatment conditions to the untreated medium controls. +, $p < 0.05$. *NS*, not significant.

PAMPs that bind and trigger TLR9. *Chlamydia* genomic DNA contains unmethylated cytosines within CpG motifs. The synthetic ODN ODN1826 is a TLR9-specific agonist. As shown in Fig. 4, ODN1826 was unable to induce secretion of IL-6 or GM-CSF in either cell line, which is consistent with the absence of TLR9 mRNA in the RT-PCR analyses. TLR9 is not likely to be an important PRM for epithelial cells during *Chlamydia* upper reproductive tract infections, at least in the murine *C. muridarum* experimental model.

TLR2 plus TLRs 1 and 6 are expressed and functional in uninfected oviduct epithelial cells

RT-PCR analysis shows that both oviduct epithelial cell lines express TLR2. TLR2 exists on cell surfaces as heterodimeric complexes with either TLR1 or TLR6. TLR1 and TLR6 do not have known PAMP recognition functions independent of TLR2 (51). TLR2 heterodimers are triggered by a wide variety of PAMPs, including peptidoglycan, lipopeptides, and fungal cell wall components. Exposure of the oviduct epithelial cell lines to the TLR2 agonist Pam_3CSK4, a lipopeptide analog, caused modest secretion of IL-6 and GM-CSF. The cytokine responses were saturated at 100 ηg/ml Pam_3CSK4 in both cell lines (Fig. 5). Pam_3CSK4 is a weak activator of TLR2 without simultaneous engagement of other signaling molecules such as dectin-1 (52). Conversely, exposure of the oviduct epithelial cell lines to *E. coli* peptidoglycan (PGN-EC) caused marked secretion of IL-6 and GM-CSF at levels similar to that seen with *C. muridarum* infection (Fig. 5).

TLR4 is not functional in oviduct epithelial cells

RT-PCR analysis did not detect TLR4 mRNA in either oviduct epithelial cell line (Fig. 3). To further investigate any possible role of TLR4 in epithelial detection of *C. muridarum*, we exposed the cell lines to "ultrapure" *E. coli* LPS. Standard preparations of LPS are commonly contaminated by other microbial TLR2 PAMPs such as lipopeptides (53). Exposure of the oviduct epithelial cell lines to 1 μg/ml ultrapure LPS caused little or no secretion of IL-6. In the same experiments, exposure of RAW 264.7 macrophage cells to 1 μg/ml ultrapure LPS triggered marked secretion of IL-6 (Fig. 6). RAW 264.7 cells express significant levels of TLR4 mRNA in RT-PCR analyses (*data not shown*). The modest amount of IL-6 released by the Bm1.11 cell line in response to ultrapure LPS is 10- to 50-fold less than the same cell line releases in response to either PGN-EC or *C. muridarum* infection (Fig. 5*A*). The IL-6 secreted by Bm1.11 cell in response to ultrapure LPS may be due to minor residual contaminants in the ultrapure LPS preparation signaling via TLR2 or Nod1 signaling pathways, or an alternative LPS signaling pathway, such as CD180 present in B cells and macrophages (54), that could exist in some reproductive tract epithelial cells.

Acute phase cytokine secretion induced by Chlamydia *infection is MyD88 dependent*

With the exception of TLR3, all TLRs trigger secretion of acute phase cytokines such as IL-6 and GM-CSF via a MyD88-dependent signaling pathway. The NBS/LRR proteins Nod1 and Nod2 trigger acute phase cytokine secretion via the RICK/RIP2/CARDIAK adaptor, a MyD88-independent signaling pathway (55). To determine whether epithelial secretion of acute phase cytokines is dependent on MyD88 signaling, Bm1.11 cells were transfected with either siRNA specific for the *MyD88* gene (siMyD88), a control scrambled RNA sequence siRNA (siSCR), or a DN MyD88 expression plasmid. To demonstrate the efficiency and specificity of the siRNA used in these experiments, real-time RT-PCR analyses were done with cytoplasmic RNA from siMyD88 and siSCR transfectants. Calculations from the SYBR-green readout of multiple real-time PCR assays demonstrated a 7.5- to 9.6-fold reduction of MyD88 mRNA in the siMyD88 transfections. Fig. 7*A* shows typical results of siRNA experiments analyzed by real-time

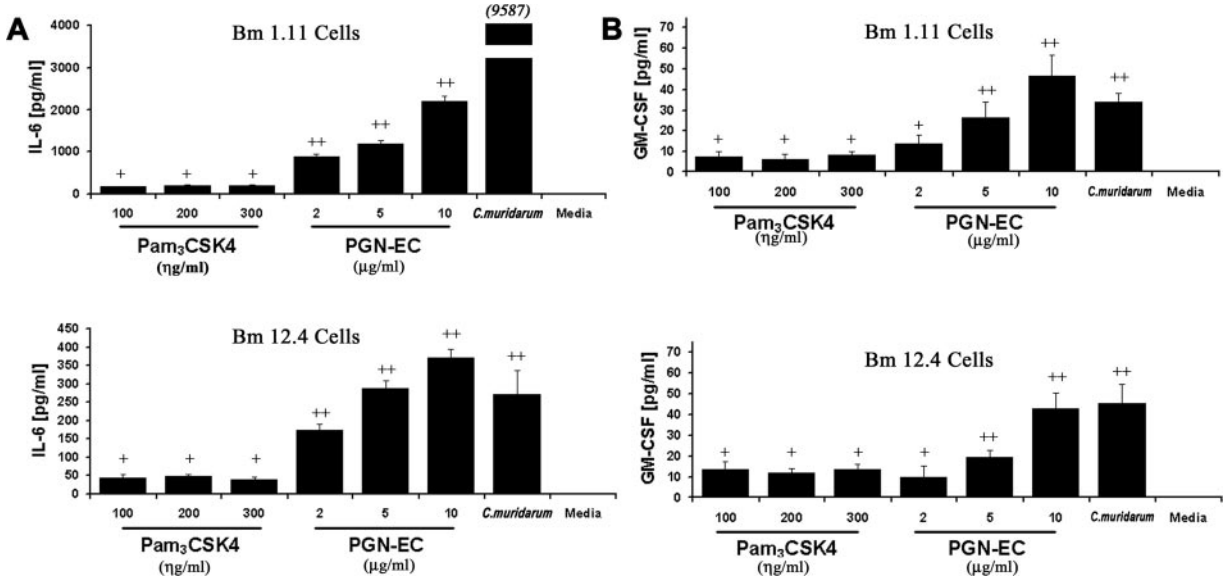

FIGURE 5. TLR2 is functional in oviduct epithelial cells. IL-6 (*A*) and GM-CSF (*B*) were secreted into the supernatants of Bm1.11 and Bm12.4 cells stimulated with the synthetic lipopeptide Pam_3CSK4 or peptidoglycan (PGN-EC). The cells were treated with the concentration ranges specified, and supernatants were collected at 30 h for analyses. The oviduct epithelial cell lines were also infected with *C. muridarum* at 10 IFU/cell (MoPn). Data presented are representative of three or more independent experiments. Statistical significance was assigned by comparing the treatment conditions to the untreated medium controls. +, $p < 0.05$; ++, $p < 0.01$.

PCR (*first panel*) and a semiquantitative two-step PCR using MyD88 specific primers (*second panel*). Fig. 7*B* shows a Western blot demonstrating expression of native MyD88 and truncated (DN) MyD88 proteins in the DN-MyD88 cell line and native MyD88 protein in Bm1.11 cells.

siMyD88-transfected, siSCR-transfected, and DN-MyD88 cells were stimulated with purified PGN-EC, infected with *C. muridarum*, or left untreated. Relative TLR activation via the MyD88 pathway was determined by measuring IL-6 released into the medium (Fig. 7*C*). As shown, transfection of the Bm1.11 cells with the siRNA specific for the *MyD88* gene, or expression of the dominant negative MyD88 protein, greatly reduced the IL-6 response to PGN-EC compared with Bm1.11 cells that were transfected with the siSCR or untreated Bm1.11 cells, respectively. Additionally, Bm1.11 cells transfected with the MyD88-specific siRNA, or expressing the DN MyD88 construct, exhibited marked reductions in IL-6 secretion when infected with *C. muridarum*

The data above are consistent with a dominant role for TLR2 in epithelial secretion of acute phase cytokines in response to *C. muridarum* infection. MyD88-dependent signaling through TLRs 4, 7, 8, and 9 is improbable because their mRNAs were not detected by RT-PCR in the oviduct epithelial cell lines. TLR5 is an unlikely trigger for acute phase cytokine secretion because *Chlamydiae* lack flagellin. The marked reduction in IL-6 secretion seen by blocking the MyD88 signaling pathway suggests that Nod1 and Nod2 are not major PRM for *Chlamydia*-infected epithelial cells because they trigger acute phase responses through a MyD88-independent signaling pathway (55).

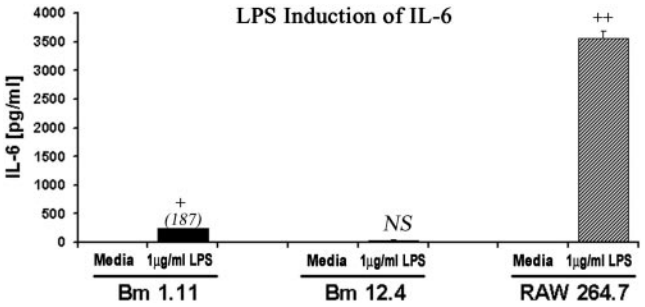

FIGURE 6. TLR4 is not functional in oviduct epithelial cells. Bm1.11, Bm12.4, and RAW 264.7 cells were simulated with 1 μg/ml ultrapure LPS. The supernatants were collected at 24 h and IL-6 levels determined. Data presented are representative of three independent experiments. Statistical significance was assigned by comparing the treatment conditions to the untreated medium controls. ++, $p < 0.01$.

TLR3 is expressed but not activated by poly(I:C) in oviduct epithelial cell lines

RT-PCR analysis (Fig. 3) detected mRNA for TLR3 in both oviduct epithelial cell lines. The natural PAMPs for TLR3 molecules are dsRNA molecules associated with RNA virus replication and possibly cellular mRNA from necrotic cells (56). TLR3 is unique among the TLRs in that it is an important TLR for IFN-β induction, and its induction of IFN-β is MyD88 independent (57). PAMP-activated TLR3 signals through an adaptor protein called Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)/Toll-IL-1R domain-containing adaptor molecule (TICAM)-1 to activate IFN regulatory factor (IRF)3 that drives transcription of IFN-β. The other major TLR signaling pathways that lead to IFN-β transcription are TLRs 4, 7, 8, and 9. TLR4 activates IRF3 via the TICAM2 and TRIF/TICAM-1 adaptor proteins (51, 58). TLR 7, 8 and 9 induce IFN-β via MyD88-dependent pathway(s) where IFN-β transcription is driven by IRF5 or IRF7 rather than IRF3 (59, 60).

We previously reported that *C. muridarum*-infected Bm1.11 cells expressed moderate levels of IFN-β mRNA in RNase protection assays as early as 12 h postinfection. In multiple experiments, we consistently detect IFN-β in culture supernatants of infected Bm1.11 and Bm12.4 cells. *Chlamydia* species have no obvious PAMP to serve as a ligand for TLR3.

As expected, blocking the MyD88 signaling pathway with MyD88-specific siRNA had minimal affects (~10% decrease) on IFN-β secretion by infected Bm1.11 cells (infected-Bm1.11:

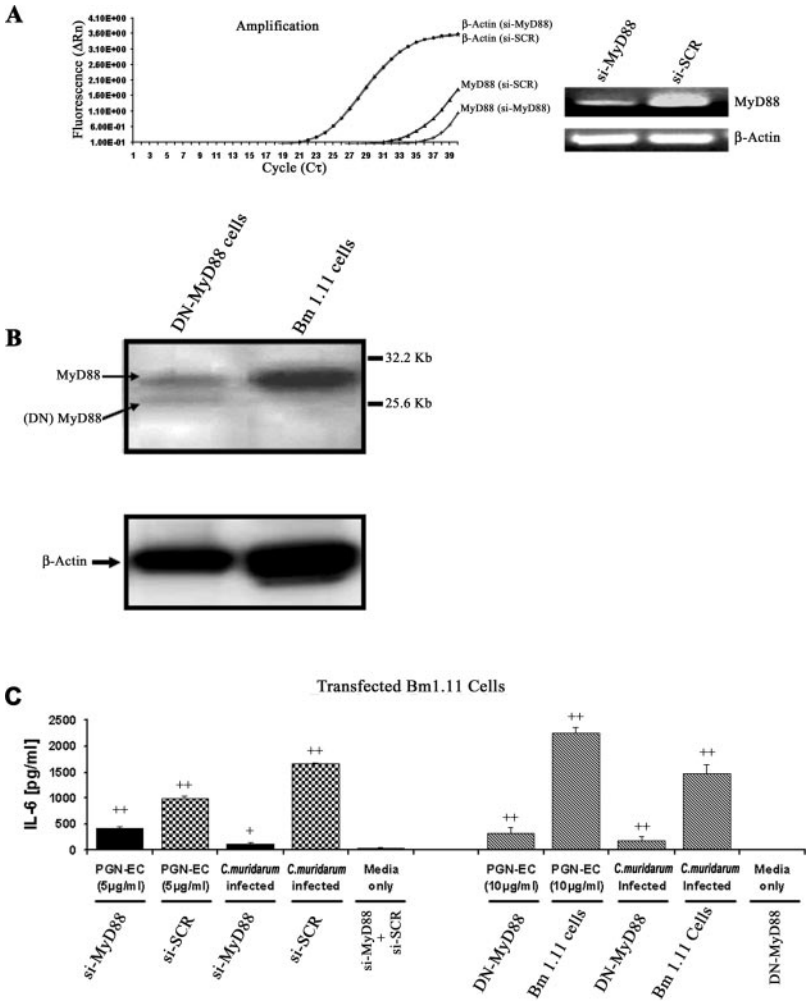

FIGURE 7. *Chlamydia* activation of oviduct epithelial lines is MyD88 dependent. *A*, Real-time RT-PCR and semiquantitative RT-PCR were performed on Bm1.11 cells transfected with either siMyD88 or siSCR. Control reactions were setup with primers specific for β-actin specific to ensure that equal amounts of template cDNA were used. *B*, Western blot analysis of MyD88 and truncated MyD88 proteins in DN-MyD88 cells and Bm1.11 control cells. Arrows indicate the endogenous "MyD88" and truncated "(DN) MyD88" proteins. *C*, Blocking the MyD88 signaling pathway using either siRNA or a truncated DN MyD88 protein diminished IL-6 secretion. Bm1.11 cells were transfected with either siMyD88 or siSCR; then the cells were either infected with 10 IFU/ml *C. muridarum* (MoPn), stimulated with 5 μg/ml PGN-EC, or not treated (siMyD88 + siSCR). Transfection of siMyD88 and siSCR did not trigger IL-6 secretion (siMyD88 + siSCR). Similarly, the DN-MyD88 and Bm1.11 control cells were stimulated with 10 μg/ml PGN-EC or infected with 10 IFU/cell of *C. muridarum* (MoPn). IL-6 levels were determined by ELISA 24 h after indicated treatments. Data presented are representative of three or more independent experiments. Statistical significance was assigned by comparing the treatment conditions to the untreated controls. +, $p < 0.05$; ++, $p < 0.01$.

197 ± 4 pg/ml; siMyD88 treated-then-infected-Bm1.11: 174 ± 3 pg/ml). Poly(I:C) is a synthetic nucleoside polymer that mimics dsRNA and is a potent TLR3 agonist. Exposure of the oviduct epithelial cells to 75 μg/ml poly(I:C) (Fig. 8), and in concentrations as high as 200 μg/ml (*data not shown*), did not trigger IFN-β secretion by the oviduct epithelial cell lines. In contrast, RAW 264.7 cells treated with poly(I:C) released substantial amounts of IFN-β into the medium in the same experiments demonstrating that the poly(I:C) stock was bioactive. *C. muridarum*-infected Bm1.11 and Bm12.4 epithelial cells, and RAW264.7 cells exposed to *C. muridarum*, secreted IFN-β (Fig. 8). Treatment of oviduct epithelial cells with the TLR3 agonist poly(I:C) also did not trigger detectable IL-6 secretion (data not shown).

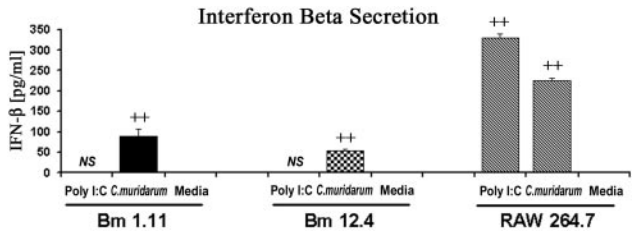

FIGURE 8. TLR3 in oviduct epithelial cells is not triggered by poly(I:C). Bm1.11, Bm12.4, and RAW 264.7 cells were either stimulated with 75 μg/ml poly(I:C), infected with 10 IFU/cell *C. muridarum*, or left untreated. After 24 h of stimulation or infection, the relative amounts of IFN-β secreted into the medium were determined. Data presented are representative of at least three independent experiments. Statistical significance was assigned by comparing the treatment conditions to the untreated medium controls. +, $p < 0.05$; ++, $p < 0.01$.

TLR3 is localized on cell surfaces in fibroblasts and intracellularly in dendritic cells. Dendritic cells with intracellular TLR3 receptors respond to poly(I:C) exposure by secreting IFN-β (61). TLR3 localization in epithelial cells is not known. It is not clear from our data whether TLR3 is the PRM responsible for IFN-β secretion by *C. muridarum*-infected epithelial cells.

Discussion

Understanding *Chlamydia*-induced pathophysiology is critical for developing interventions to prevent tubal infertility and ectopic pregnancies. Fallopian tube epithelium is the logical epicenter for the relevant pathophysiology because 1) *Chlamydia* replication is restricted almost exclusively to reproductive tract epithelial cells, and 2) Fallopian tube epithelium lines the lumen of the reproductive organ that becomes distorted and dysfunctional as a consequence of the infection. Our work and that of others have previously shown that *Chlamydia*-infected epithelial cells secrete proinflammatory cytokines (14–19), including cytokines that have roles in leukocyte recruitment, leukocyte activation, fibrosis, and scarring. In this report, we address the PRMs that trigger cytokine release by infected oviduct epithelial cells. To our knowledge this is the first investigation focused on oviduct epithelial cells.

Our murine oviduct epithelial cell lines express TLRs 1, 2, 3, 5, and 6 as determined by RT-PCR analysis. With respect to TLR4,

this expression pattern is similar to that described for primary and immortalized human lower reproductive tract epithelial cell lines derived from ectocervix and endocervix that lack TLR4. Similar to the primary and immortalized cervical epithelial cell lines, our oviduct epithelial cell lines were not significantly activated by *E. coli* LPS, a potent TLR4 ligand (34). Conversely, our murine oviduct epithelial lines differ significantly from a human uterine epithelial cell line and primary uterine epithelial cell lines that express TLR4 in addition to TLRs 1, 2, 3, 5, 6, 7, 8, and 9 (35, 37). The discordant TLR4 findings may represent intrinsic differences between epithelial cells lining the uterus vs those lining the cervix and oviducts. The absence of TLRs 7–9 in our mouse oviduct epithelial cell lines may represent intrinsic differences related to localization within the reproductive tract or differences between mice and humans.

At least in the murine system, TLR4 does not appear to play a major role in oviduct epithelial secretion of acute phase cytokines, such as IL-6 and GM-CSF, in response to *C. muridarum* infection. This conclusion is consistent with data from experiments done in TLR4-knockout mice. Mice deficient in TLR4 are not compromised in their ability to clear *C. muridarum* infections and are not spared the deleterious scarring of the oviducts caused by infection. TNF-α, IL-6, IFN-γ, and MIP-2 levels in genital secretions of infected TLR4-deficient mice are nearly identical to levels seen in wild-type mice (38).

Based on RT-PCR expression analysis, TLRs 2, 3, and 5 and the NBS/LRR proteins Nod1 and Nod2 were candidate PRMs for oviduct epithelial cell activation by *Chlamydia* infection. The inability to detect mRNA for TLRs 4, 7, 8, and 9 by RT-PCR analysis eliminated those TLRs as likely *Chlamydia* PRM for oviduct epithelial cells. However, while the Bm1.11 and Bm12.4 epithelial cell lines express TLR5, *E. coli* flagellin only weakly activated cytokine secretion and the *Chlamydia* genome does not encode a flagellin. TLR5 is not likely to be an important TLR for oviduct epithelial activation by *Chlamydia* infection, although it may play a role in polymicrobial pelvic inflammatory disease.

TLR3 is expressed by the epithelial cell lines and has a MyD88-independent pathway using TRIF/TICAM-1 instead of MyD88 to activate TRAF6 leading to NF-κB activation and acute phase cytokine secretion (62). Treatment of the oviduct epithelial cell lines with poly(I:C) did not trigger detectable IL-6 secretion. Our data differ from primary human polarized uterine epithelial cells that secreted significant amounts of IL-6, GM-CSF, TNF-α, and G-CSF in response to poly(I:C) (36). It is not clear whether the difference observed is due to a species difference, polarization vs monolayer culture, or intrinsic differences in epithelial cells lining the uterus vs those lining the oviducts. At least in murine oviduct epithelial cells, the dependence of IL-6 secretion on MyD88 and the lack of IL-6 secretion in response to the TLR3 agonist poly(I:C) make it unlikely that TLR3 makes a major contribution toward secretion of acute phase cytokines by infected oviduct epithelial cells.

Dependence of acute phase cytokine secretion on MyD88 signaling identifies TLR2 heterodimeric complexes as the principal TLR activating oviduct epithelial cell lines during *C. muridarum* infection. siRNA transfection targeting MyD88 and expression of a truncated DN MyD88 protein had dramatic effects on reducing IL-6 secretion triggered by the TLR2 agonist peptidoglycan or by *Chlamydia* infection. TLR2 signaling is MyD88 dependent (51). Because IL-6 secretion was MyD88 dependent and Nod1/Nod2 signaling is MyD88 independent, Nod1 and Nod2 are not likely to play major roles in oviduct epithelial secretion of acute phase cytokines in response to infection.

The conclusion that TLR2 heterodimers are the principal TLR for epithelial acute phase cytokine responses to infection is consistent with data from TLR2-knockout mice. Like TLR4-deficient mice, TLR2-deficient mice clear *Chlamydia* genital infections with kinetics similar to wild-type controls. Unlike TLR4-deficient mice, TLR2-deficient mice infected by *C. muridarum* had significantly lower levels of TNF-α, IL-6, IFN-γ, and MIP-2 in genital secretions compared with wild-type controls, implying a major role for TLR2 in releasing cytokines during infections of the genital tract. In addition, TLR2-deficient mice develop less oviduct scarring than is seen in infected wild-type and TLR4-deficient mice. TLR2 appears to contribute to oviduct pathology without contributing to host defense (38). Our data complement the in vivo data of Darville et al. (38) and suggest that TLR2 on oviduct epithelial cells contributes to acute phase cytokine secretion and scarring.

We have not investigated the *Chlamydia* PAMPs responsible for epithelial activation via TLR2. The recent finding that *C. trachomatis* LPS activates NF-κB by signaling through TLR2 rather than TLR4 (63) and that *C. trachomatis* has a potential peptidoglycan synthesis pathway (64, 65) suggest several possible *Chlamydia* PAMPs for TLR2.

We attempted to determine the PRM responsible for oviduct epithelial secretion of the immunomodulatory cytokine IFN-β. IFN-β plays an important role in the transition from innate to adaptive immunity (66). We previously showed that IFN-β mRNA is up-regulated in oviduct epithelial cells infected with *C. muridarum* (14). We document that infected oviduct epithelial cells secrete IFN-β in this report. Engagement of TLRs 3, 4, 5, 7, 8, and 9 up-regulates transcription of IFN-β via several different signaling pathways (51, 57–59). However, IFN-β induction via TLR5 is TLR4 dependent (67), and TLRs 4, 7, 8, and 9 were not expressed by our oviduct epithelial lines. Although TLR3 is the most logical TLR candidate for IFN-β secretion by infected-oviduct epithelial cells, we were not able to demonstrate that TLR3 was functional in the oviduct epithelial cell lines using the TLR3 agonist poly(I:C). This result differs from data with polarized human primary uterine epithelial cell lines that up-regulate IFN-β mRNA in response to poly(I:C) (36). It is unclear whether this observed difference is due to species, culture conditions, or differences between uterine and oviduct epithelium. The above issue aside, *Chlamydiae* do not have a known dsRNA type PAMP recognizable by TLR3. Cellular mRNA associated with necrotic cells has been shown to be a TLR3 ligand in dendritic cells (56); however, cellular mRNA is a less potent TLR3 agonist than poly(I:C), and IFN-β is up-regulated before cell death occurs in the oviduct epithelial cell cultures (data not shown). Currently, it is not clear whether TLR3 plays a significant role in IFN-β secretion by *Chlamydia*-infected oviduct epithelial cells. It is possible that *C. muridarum* induces IFN-β through an alternative pathway such as the IRF-3-dependent, TLR-independent, and Nod2-independent pathway described in macrophages infected by *Listeria monocytogenes* (68).

In vitro experiments using the cloned oviduct epithelial cell lines Bm1.1 and Bm12.4 implicate TLR2 as the principal PRM responsible for acute phase cytokine secretion triggered by *C. muridarum* infection. These cell lines should serve as useful reagents for determining the PRM responsible for infection-induced secretion of immunomodulatory cytokines such as IFN-β. Based on agreement with previously published in vivo data in TLR2- and TLR4-knockout mice, the oviduct epithelial cell lines appear to be useful reagents for studying innate epithelial responses to *Chlamydia* infection in the murine model.

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Disclosures

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