Chlamydia muridarum Infection Elicits a Beta Interferon Response in Murine Oviduct Epithelial Cells Dependent on Interferon Regulatory Factor 3 and TRIF

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Received 21 September 2006/Returned for modification 23 October 2006/Accepted 11 December 2006

Chlamydia trachomatis is the most common sexually transmitted bacterial infection in the United States. Utilizing cloned murine oviduct epithelial cell lines, we previously identified Toll-like receptor 2 (TLR2) as the principal epithelial pattern recognition receptor (PRR) for infection-triggered release of the acute inflammatory cytokines interleukin-6 and granulocyte-macrophage colony-stimulating factor. The infected oviduct epithelial cell lines also secreted the immunomodulatory cytokine beta interferon (IFN-β) in a largely MyD88-independent manner. Although TLR3 was the only IFN-β production-capable TLR expressed by the oviduct cell lines, we were not able to determine whether TLR3 was responsible for IFN-β production because the epithelial cells were unresponsive to the TLR3 ligand poly(I-C), and small interfering RNA (siRNA) techniques were ineffective at knocking down TLR3 expression. To further investigate the potential role of TLR3 in the infected epithelial cell secretion of IFN-β, we examined the roles of its downstream signaling molecules TRIF and IFN regulatory factor 3 (IRF-3) using a dominant-negative TRIF molecule and siRNA specific for TRIF and IRF-3. Antagonism of either IRF-3 or TRIF signaling significantly decreased IFN-β production. These data implicate TLR3, or an unknown PRR utilizing TRIF, as the source of IFN-β production by Chlamydia-infected oviduct epithelial cells.

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Published ahead of print on 18 December 2006.
blasts TLR3 has been reported to be expressed on the cell surface (41). It is not known where TLR3 localizes in murine reproductve tract epithelial cells, but intracellular localization in pulmonary epithelial cells and the lack of a murine oviduct epithelial response to extracellular poly(I-C) supports an endosomal localization. Known ligands for TLR3 include viral double-stranded RNA (dsRNA) (2) and possibly cellular RNA (27).

Signal transduction by TLR3 is mediated via TRIF (also known as Ticam-1) (48, 64, 66). Ligand-activated TLR3 recruits TRIF via its intracellular TIR domain. TRIF recruits a TANK-containing complex (9, 18, 50, 54) that phosphorylates IRF3. Phosphorylated IRF3 dimerizes and translocates to the nucleus, where it initiates transcription from the IFN-β promoter (59). The IFN-β promoter also has NF-κB and AP-1 binding sites; however, these sites are less critical for IFN-β transcription (51). The known TLR3 signaling pathway to IFN-β production is dependent on TRIF and IRF3. The lack of TLR4 expression makes TLR3 the only known TRIF-dependent TLR in murine oviductal epithelial cell lines (8).

CARD family members RIG-I and MDA5 are resident in the cytosol and likely to play a role in host defense against some RNA viruses (3, 68). Like TLR3, RIG-I and MDA5 recognize dsRNA (25, 60, 68). RIG-I and MDA5 signal through an adaptor variously known as IPS-1, MAV, VISA, and Cardif (reviewed in reference 23). IPS-1 serves a TRIF-like role for RIG-I and MDA5, connecting these PRRs to the IKKe/TBK1 activation pathways for IRF3 and to the TRAF6/IKKe pathways for the activation of NF-κB. Most studies of RIG-I and MDA5 signaling support a TRIF-independent signaling pathway for IFN-β production (28, 29, 43, 53, 55), although one study did not (63). Mice deficient in IPS-1 are markedly deficient in their IFN-β response to RNA viruses or transfected poly(I-C), thereby identifying IPS-1 as the sole adaptor for RIG-I and MDA5 (30). The putative cytosol-resident DNA PRR is also IPS-1 dependent (21).

For our purposes, investigation of the IRF3 and TRIF signaling pathways would potentially identify the PRR responsible for IFN-β production. RIG-I, MDA5, the putative cytosolic DNA PRR, and TLR3 are IRF3 dependent for IFN-β production. Of these candidate PRRs, only TLR3-mediated production of IFN-β would be dependent on the TRIF adaptor. TRIF dependence would implicate TLR3, whereas TRIF independence would implicate RIG-I/MDA5/cytosolic DNA PRR as the PRR responsible for IFN-β production by C. muridarum-infected oviductal epithelial cells.

**MATERIALS AND METHODS**

**Reagents.** Poly(I-C), product number P-0913, was purchased from Sigma Chemical Co. (St. Louis, MO).

**Cells, plasmids, and bacteria.** Derivation and maintenance of the cloned oviduct epithelial cell line Bm1.11 has previously been described (8, 24). The dominant-negative MyD88 expression plasmid pRES2-EGFP-DM-MyD88 and the (DN)MyD88 cell line were previously described (8). The dominant-negative TRIF expression plasmid pcDNA-TRIF-TIR encoding TRIF amino acids 397 to 530 with a C-terminal His tag was cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) using the forward primer 5′-CAACACTGTAT AACCTTTGGTTATCCGGGACG3′ and the reverse primer 5′-GCTGTTGGTTGACCTTTCTGCGAAGATTGGGGA-3′. Bm1.11 cells were transfected with the pcDNA-TRIF-TIR, and two independently derived cell lines designated (DN)TRIF1 and (DN)TRIF2 were derived by limiting dilution (see below). Two cloned cell lines were kept to rule out clone-specific artifacts.

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 previously described (8).

**Transfections.** To generate the dominant-negative TRIF-expressing clones (DN)TRIF1 and (DN)TRIF2 or the pcDNA3.1D control cell line, 75% confluent Bm1.11 cells in six-well plates were transfected with 5 μg of either the pcDNA-TRIF-TIR plasmid or the pcDNA3.1D His parent vector using Lipofectamine 2000 reagent (Invitrogen). The DNA-Lipofectamine 2000 complexes were incubated for 30 min in serum-free Dulbecco modified Eagle medium at room temperature, and the complexes were added to the Bm1.11 monolayers, followed by incubation 5 h in a 37°C CO2 incubator. After 5 h of incubation, the transfection medium was replaced with fresh epithelial cell medium and incubated an additional 30 h at 37°C. The dominant-negative TRIF and empty vector control cells were selected in epithelial media supplemented with 800 μg of G418/ml. The (DN)MyD88 and (DN)TRIF cell lines were maintained in epithelial-cell media supplemented with 400 μg of G418/ml. Transient transfections were performed with 10 μg of either of the pcDNA-TRIF-TIR plasmid or the pcDNA3.1D-His control vector in Lipofectamine 2000.

**Poly(I-C) treatment.** Poly(I-C) was added directly to the medium of Bm1.11, (DN)TRIF1, and (DN)TRIF2 cells and the pcDNA3.1D control cells at 50, 75, and 100 μg/ml depending on the experiment. Supernatants were assayed for poly(I-C)-induced IFN-β responses by enzyme-linked immunosorbent assay (ELISA) 24 h after transfection. Transfections of poly(I-C) were done by complexing either 50 or 75 μg/ml to Lipofectamine 2000 reagent in serum-free media as previously described (36). After a 30-min incubation at room temperature, the poly(I-C) complexes were added to Bm1.11, (DN)TRIF1, and (DN)TRIF2 cells and the pcDNA3.1D control cells for 5 h as described above. After 5 h, the transfection medium was removed and replaced with fresh epithelial cell media.

**Infections.** Bm1.11, (DN)MyD88, (DN)TRIF1, and (DN)TRIF2 cell lines were plated in 48-well tissue culture plates and were used when confluent (104 cells/well). For all experiments, the cells were infected with 10 inclusion forming units (IFU) of C. muridarum per well in 900 μl of culture medium. The 48-well plates were centrifuged at 1,000 rpm (300 g) for 1 h at 30°C, incubated 30 h, and then incubated at 500 g for 1 h at 37°C in a 5% CO2 humidified incubator without subsequent change of medium for 6 to 30 h, depending upon the assay. Mock-infected wells received an equivalent volume of sucrose-phosphate-glutamic acid buffer lacking C. muridarum.

For experiments with the endosomal acidification inhibitor bafilomycin A (Sigma), epithelial cells were infected with 10 IFU per cell with 1 h of centrifugation followed by 2 h of incubation at 37°C prior to the addition of bafilomycin in the indicated concentrations. The bafilomycin A stock solution was prepared in dimethyl sulfoxide (DMSO). Comparator “untreated” wells received an equivalent amount of the DMSO vehicle.

**Western blotting.** Control and (DN)TRIF expressing Bm1.11 cells were grown in monolayers in a six-well plate to confluence. After removal of the growth medium, the monolayers were gently washed with phosphate-buffered saline, and cytosolic proteins were recovered by the cell fractionation performed in the PARIS kit (Ambion, Austin, TX). Cytosolic proteins were quantified by using the Micro BCA protein assay kit (Pierce, Rockford, IL). Then, 25 μg of lyaste from either the (DN)TRIF1, (DN)TRIF2, or nontransfected Bm1.11 cell line was boiled in 5× Immunopore reducing sample buffer (Pierce) prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After separation by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, the proteins were transferred to Immobilon-P (Millipore, Bedford, MA) transfer membranes. Transfer membranes were blocked in 5% nonfat dry milk, and subsequent immunoblotting was performed by using a 1:5,000 dilution of the His tag-specific rabbit polyclonal antibody SC-804 (Santa Cruz Biotechnology, Santa Cruz, CA) with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (Amersham Biosciences, Piscataway, NJ). Proteins were visualized via chemiluminescence using the ECL plus Western blotting detection system (Amersham) as described in the manufacturer’s protocol.

**RNA interference.** MyD88-specific and IRF3-specific small interfering RNA (siRNA), MyD88- and IRF3-specific primers, scrambled control siRNA, siRNA transfection reagent, and siRNA transfection medium were all purchased from Santa Cruz Biotechnology as proprietary reagents. Custom-designed TRIF-specific siRNA and primers were purchased from Ambion (Table 1). siRNA transfections targeting endogenous MyD88, TRIF, IRF3, and scrambled controls were carried out in 48-well plates with 4 × 10⁴ Bm1.11 cells per well using the manufacturer’s protocol. The siRNA cocktail was mixed with the transfection reagent mix and allowed to form liposome-siRNA complexes for 30 min at room temperature before being added to the cells. After an initial 5 h of incubation, the transfection medium was replaced with fresh epithelial medium, and the cells
were returned to the 37°C CO₂ humidified incubator for 24 to 48 h before we used them in experiments.

**RT-PCR.** Total RNA was isolated from dominant-negative cell lines and the Bm1.11 oviduct epithelial control cells by using RNaseasy minicolumns (Qiagen, Valencia, CA). 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, and RNA integrity was confirmed by agarose gel electrophoresis. Optimized primer pairs were designed by using the Vector NTI Suite (InforMax, Inc., Frederick, MD). The specific primer pairs (Amitoff, Alston, MA) are listed in Table 1. 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, Madison, WI). The cycling conditions were as follows: 1 min and 30 s of initial denaturation at 95°C, followed by eight cycles of 30 s at 95°C, 15 s at 56°C, and 30 s at 72°C. After the initial 8 cycles, the 30-s 72°C extension cycle was increased 3 s per cycle for 31 cycles. During the 40th cycle, the 72°C extension was 3 min to complete the RT-PCR. Reactions were also amplified in the absence of reverse transcriptase as negative controls.

**Real-time RT-PCR.** Cytoplasmic RNA was purified from the siRNA transfected Bm1.11 cells by 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, Hercules, CA). The cDNA product was diluted 1:50, whereas the MyD88-specific, IRF3-specific, TRIF-specific, and β-actin control primers (Table 1) were adjusted to 2 pmol/μl working stock. Real-time PCR was conducted with the diluted cDNA and primers in accordance with the protocol outlined in the iTaq SYBR green Supermix with ROX kit (Bio-Rad). Real-time PCR was performed with an ABI Prism 7000 machine (Applied Biosystems, Foster City, CA): 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 2 min at 60°C. Cycle threshold (Ct) 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 (–ΔCt), where E is the amplification efficiency and m is the slope of the dilution curve. Dissociation curves were recorded after each run to ensure primer specificity.

**ELISA determination of cytokine production.** Confluent Bm1.11, (DN)TRIF, and (DN)MyD88 monolayers grown in 48-well tissue culture-treated plates were either infected with 10 IFU of *C. muridarum* per cell or pre-treated with siRNA transfection prior to infection. Supernatants were harvested at 18 h or 24 h (depending upon experiment) and analyzed by ELISA for IFN-β as previously described (8). All standards and experimental samples were analyzed in triplicate. The lower range of assay sensitivity for IFN-β was 10 pg/ml.

**Statistical analyses.** Summary figures for each experimental investigation are presented as a “pooled” means and with their associated standard deviations. Figure legends indicate the number of independent experiments pooled to generate each figure. Analysis of variance models with one or two fixed effect factors, including the two-way interaction if significant, considered the time of the experiment or the run as a random block effect to account for the correlation of observations measured within the same experimental investigation. The tests of lack of fit and model assumptions of homogeneity of variance and normality suggested a log transformation for the *Chlamydia* growth response (see Fig. 7). Each fixed factor or group effect was tested, and the group means were compared. The Tukey-Kramer adjustment method for multiple comparisons was used to control the type I error. Statistical analyses were performed by using the statistical software package SAS version 9.1 (SAS Institute, Cary, NC).

## RESULTS

*C. muridarum* infection induces an IFN-β response by Bm1.11 oviduct epithelial cells. We previously reported that *C. muridarum* infection induced IFN-β mRNA and IFN-β protein synthesis in Bm1.11 oviduct epithelial cells (8, 24). Consistent with our previous reports, IFN-β secretion by infected Bm1.11 cells was detectable by 12 h postinfection and increased steadily through the 24 h time point (Fig. 1A). Bm1.11 cells at 18 h postinfection produced a level of IFN-β intermediate between that seen at the 12- and 24-h time points (data not shown). IFN-β secretion was dependent on active *Chlamydia* replication since Bm1.11 cells exposed to heat-killed *C. muridarum* did not secrete levels of IFN-β statistically different from mock-infected cells (Fig. 1B).

**Bm1.11 oviduct epithelial cells express TLR3, RIG-I, and MDAs but not TLR4, TLR7, TLR8, and TLR9.** PRR signaling pathways that result in IFN-β production are MyD88-dependent (TLR7 to TLR9) or MyD88-independent (TLR3, TLR4, RIG-I, and MDAs). RT-PCR analysis was performed on Bm1.11 total RNA for the PRRs relevant to IFN-β production. Consistent with our previous study, the Bm1.11 oviduct epithelial cells have mRNA for TLR3 but lack mRNA for TLR4 and TLR7 to TLR9 (Fig. 2A) (8). Bm1.11 cells have mRNA for RIG-I and MDAs (Fig. 2B). Bm1.11 cells also have mRNA for TLR11 (data not shown), a recently discovered TLR that signals through MyD88 (67, 69).

**IFN-β secreted by the oviduct epithelial cells infected with *Chlamydia* requires IRF3.** The transcription factor IRF3 is required for the induction of IFN-β through TLR and CARD pathways. We utilized siRNA technology to examine the effect of lowering IRF3 mRNA levels on IFN-β secretion by *C. muridarum*-infected Bm1.11 cells. Transfection of Bm1.11 cells with IRF3-specific siRNA in individual experiments resulted in a 3.23- to 3.84-fold reduction in IRF3 mRNA 24 to 30 h posttransfection compared to scrambled siRNA-transfected controls (si-SCR) as determined by real-time RT-PCR (Fig. 3A). As shown in Fig. 3B, lowering IRF3 mRNA levels with si-IRF3 resulted in a reduction of ca. 60% in IFN-β secretion.

### Table 1. Primers used for RT-PCR and siRNA

| Product | Primer sequence (5'-3') | PCR product size (bp) |
|---------|-------------------------|----------------------|
| TLR3    | ACCCTTCAAAAAACAGAAAGATC | 521                  |
| TLR4    | TCAACCCCTTGAAGACACTAA   | 459                  |
| TLR7    | AACCACAATTACCAAGCATTTC  | 458                  |
| TLR8    | CAGAATTGGTTGATTAAGAGAGA | 477                  |
| TLR9    | GCCTGAGACCAACAACATCTCC  | 458                  |
| si-TRIF | GCUAGUGUAACACAGCCAGGT   | NA                   |
| MDA5    | TCTCTGGGTGATTCTCCTGCCA   | 458                  |
| RIG-I   | CAAAACACACCACATACATCG   | 502                  |
| β-Actin | ATGGGATTGACATGACTACGAC  | 400                  |
| MyD88   | ATCCGGGTCTTGTGTCTCTCAT  | 488                  |
| TRIF    | ATGGGATACCCAGGGCCTTCGC  | 480                  |
| IRF-3   | TGGGACGAGAGCGACAGGTTG   | 516                  |

* NA, not applicable.
24 h post *C. muridarum* infection compared to si-SCR controls. Control si-SCR RNA augmented IFN-β production by infected Bm1.11 cells, a finding consistent with the reported nonspecific siRNA induction of IFN-β pathways (26). si-IRF3-treated Bm1.11 cells also produced significantly less IFN-β than untreated Bm1.11 cells, although the appropriate experimental comparison is between the si-SCR control and si-IRF3. Partial knockdown of IRF3 levels using siRNA caused a significant decrease in IFN-β production by *C. muridarum*-infected Bm1.11 oviduct epithelial cells, thereby identifying IRF3 as a component of the PRR signaling pathway responsible for IFN-β production.

**IFN-β secretion by infected Bm1.11 oviduct epithelial cells is dependent on TRIF.** TLR3 signaling for IFN-β production is dependent on TRIF, whereas RIG-I/MDA5/cytosolic DNA PRR signaling for IFN-β production is TRIF independent. We used siRNA to determine the role of TRIF in IFN-β production by infected Bm1.11 cells. Transfection of Bm1.11 cells with TRIF-specific siRNA resulted in a 2.89- to 4.56-fold reduction in TRIF mRNA 24 h posttransfection compared to si-SCR transfected cells as determined by real-time RT-PCR (Fig. 4A). Figure 4B shows the results for experiments using siRNA to decrease levels of TRIF mRNA prior to...
FIG. 4. The IFN-β response elicited by *Chlamydia muridarum* infection is largely dependent on TRIF. (A) Quantitative real-time PCR was performed on cytoplasmic RNA isolated from Bm1.11 cells transfected with either TRIF-specific siRNA (si-TRIF) or the scrambled control siRNA (si-SCR) at 24 to 48 h posttransfection. Control reactions were set up with primers specific for β-actin to ensure that equal amounts of template cDNA were used. The representative data presented, from one of four independent experiments, show a 4.56-fold reduction in TRIF mRNA in the si-TRIF transfected cells at 48 h posttransfection. (B) Bm1.11 cells were transfected with either si-TRIF or the si-SCR control oligonucleotides and then infected with 10 IFU of *C. muridarum* per cell at 24 to 48 h posttransfection. IFN-β levels were determined 24 h postinfection. Untreated Bm1.11 cells and Bm1.11 cells transfected with si-TRIF were compared to Bm1.11 transfected with the scrambled control, si-SCR. Pooled data from five independent experiments are shown. ***** P < 0.0001; NS, not significant. (C) Western blot analysis of (DN)TRIF cell lines. Total cell lysates from the dominant-negative TRIF cell lines (DN)TRIF1 and (DN)TRIF2, the control pcDNA3.1D-His cell line, and untreated Bm1.11 cells were evaluated for the expression of the His-tagged DN-TRIF protein. Asterisks indicate the truncated His-tagged DN-TRIF protein; arrows show the control GAPDH protein. (D) The dominant-negative TRIF-expressing oviduct epithelial cell lines (DN)TRIF1 and (DN)TRIF2 and vector control cell line pcDNA3.1D were either mock treated or infected with 10 IFU of *C. muridarum* per cell. Supernatants were collected at 18 h postinfection, and IFN-β levels were measured. (DN)TRIF cell lines and untreated Bm1.11 cells were compared to the vector control cell line pcDNA3.1D. Pooled data from five independent experiments are shown. ***** P < 0.0001; NS, not significant. (E) Bm1.11 cells were transiently transfected with 10 μg of either the dominant-negative TRIF expressing plasmid pcDNA-TRIF-TIR or the pcDNA3.1D/V5-His parent vector and allowed to incubate at 37°C for 40 to 48 h prior to infection with 10 IFU of *C. muridarum* per cell. Bm1.11 cells (mock infected and infected) and pcDNA-TRIF-TIR transfectants were compared to pcDNA3.1D/V5-His parent vector control transfectants at 24 h postinfection. Pooled data from two independent experiments are shown. ** P < 0.01; ***** P < 0.0001.
infection with *C. muridarum*. Bm1.11 cells transfected with TRIF-specific siRNA 24 h prior to infection had a decrease in IFN-β production of up to 10-fold in individual experiments compared to the si-SCR and untransfected Bm1.11 controls.

To further examine the role of TRIF in the IFN-β response of *C. muridarum*-infected Bm1.11 cells, we derived cloned Bm1.11 cell lines expressing a dominant-negative form of the TRIF protein consisting of only its TIR domain. Western blot analysis demonstrated expression of His-tagged (DN)TRIF by two cloned Bm1.11 cell lines, designated (DN)TRIF1 and (DN)TRIF2, but not untransfected Bm1.11 cells or Bm1.11 cells transfected with the empty parent vector (Fig. 4C). As shown in Fig. 4D, *C. muridarum*-infected (DN)TRIF1 and (DN)TRIF2 cell lines secreted significantly lower amounts of IFN-β compared to the control Bm1.11 cell line transfected with empty vector designated pcDNA3.1D and untransfected Bm1.11 cells. *C. muridarum* replicated equally well in control pcDNA3.1D cells and the (DN)TRIF2 cell line (data not shown); *C. muridarum* replicated better (1 log greater) in the (DN)TRIF1 cell line than either the control pcDNA3.1D cells or the (DN)TRIF2 cell line (data not shown). The relative levels of *C. muridarum* replication did not explain the decrease in IFN-β secretion seen in the dominant-negative TRIF cell lines. To address the possibility that transfection or selection of a Bm1.11 cell line expressing a (DN)TRIF cell line may have altered its IFN-β response to *C. muridarum* infection, we also performed transient transfections of Bm1.11 cells with 10 μg of the dominant-negative TRIF plasmid (pcDNA-TRIF-TIR) or 10 μg of the parent pcDNA3.1D/V5-His plasmid. As shown in Fig. 4E, the level of IFN-β secreted into the medium of pcDNA-TRIF-TIR transiently transfected cells was significantly lower than control transfected and untransfected Bm1.11 cells. These data are consistent with the results obtained with the TRIF-specific siRNA and dominant-negative TRIF cell lines and therefore identify a major role for TRIF-mediated signaling in the epithelial IFN-β response to *C. muridarum* infection.

The IFN-β response to *C. muridarum* infection of the oviduct epithelial cells is largely independent of MyD88. We previously demonstrated major roles for TLR2 and MyD88 in infected oviduct epithelial secretion of the inflammatory cytokines IL-6 and granulocyte-macrophage colony-stimulating factor and showed that the majority of IFN-β secretion by infected Bm1.11 cells was MyD88 independent (8). Real-time PCR analyses of MyD88-specific siRNA treatment showed a 7.5- to 9.6-fold reduction in the MyD88 mRNA (Fig. 5A). Consistent with our previously published data, MyD88-specific siRNA suppression of MyD88 mRNA levels caused a significant 15 to 20% decrease in IFN-β secretion by infected Bm1.11 cells (Fig. 5B). In Fig. 5C and D, we compared IL-6 and IFN-β responses of the dominant-negative MyD88-expressing Bm1.11 cell line designated (DN)MyD88 with the newly derived (DN)TRIF1 and (DN)TRIF2 cell lines. *C. muridarum*-infected (DN)MyD88 cells showed a small but statistically significant decrease (range, 10 to 20%) in the amount of IFN-β secreted into the medium compared to infected Bm1.11 control cells at 24 h postinfection, whereas the majority of IFN-β production at 24 h postinfection was suppressed in the (DN)TRIF1 and (DN)TRIF2 cell lines. Conversely, the (DN)MyD88 cell line showed a >85% decrease in IL-6 secretion upon infection, while the (DN)TRIF1 and (DN)TRIF2 cell lines secreted IL-6 at levels similar to Bm1.11 control cell lines. *C. muridarum* replicated equally well in pcDNA3.1D and the (DN)MyD88 cell lines (data not shown). These data are consistent with a major role for TRIF and a lesser role for MyD88 in IFN-β secretion by infected oviduct epithelial cell lines.

**Bm1.11 cells respond to transfected poly(I-C) but not extracellular poly(I-C).** RT-PCR analysis in Fig. 2 showed that Bm1.11 oviduct epithelial cells express RIG-I and MDA5. To test whether RIG-I/MDA5 were functional in Bm1.11 epithelial cells, poly(I-C) was delivered externally or transfected into the cytoplasm, and the IFN-β response was measured (Fig. 6A). Consistent with our previously published data, extracellular poly(I-C) did not trigger detectable IFN-β secretion by Bm1.11 cells even though TLR3 mRNA can be detected by RT-PCR analysis. Conversely, transfected poly(I-C) caused marked IFN-β secretion by Bm1.11. These data are consistent with functional RIG-I/MDA5 activation and signaling in oviduct epithelial cells. To test whether this signaling was TRIF dependent, Bm1.11, (DN)TRIF1, (DN)TRIF2, and pcDNA3.1D cells were treated with extracellular poly(I-C), transfected with poly(I-C), or exposed to transfection vehicle alone (Fig. 6B). (DN)TRIF1 and (DN)TRIF2 cell lines responded to transfected poly(I-C) similar to Bm1.11 cells and the pcDNA3.1D control cell line. There was no IFN-β response to extracellular poly(I-C). The straightforward interpretation of these results is that Bm1.11 cells have functional RIG-I/MDA5 receptors in their cytoplasm and that the RIG-I/MDA5 signaling pathway to IFN-β production is independent of TRIF.

Bafilomycin A inhibits *C. muridarum* replication in infected Bm1.11 cells more profoundly than IFN-β production. Activation of endosomal TLR3 and TLR9 by their microbial ligands requires acidification that can be blocked with bafilomycin. We previously reported that bafilomycin A, an endosomal H+ proton pump inhibitor (1, 7, 38), Bafilomycin A does not interfere with *C. trachomatis* serovar L2 replication in Vero cells (17). Because Bm1.11 cells do not express TLR7 to TLR9, IFN-β production susceptibility to bafilomycin inhibition during *C. muridarum* infection would implicate TLR3 as the relevant PRR. We therefore infected Bm1.11 cells and subsequently exposed them to bafilomycin A or control medium containing the bafilomycin A vehicle DMSO. While bafilomycin had a minimal inhibitory effect on IL-6 secretion (Fig. 7A), it potently suppressed IFN-β production whether added 1 h postinfection (data not shown) or 3 h postinfection (Fig. 7B). However, bafilomycin A blocked *C. muridarum* replication more profoundly than it suppressed IFN-β production (Fig. 7C). The organic solvents used to dissolve bafilomycin, DMSO (Fig. 7) and ethanol (data not shown) had no significant effect on *C. muridarum* replication or IL-6 production. For reasons that are not clear, the DMSO (Fig. 7) and ethanol vehicles (data not shown) augmented IFN-β by *Chlamydia*-infected epithelial cells. It is unclear whether bafilomycin inhibition of endosomal acidification blocked IFN-β production directly through preventing TLR3 activation or secondarily by inhibiting *C. muridarum* replication.
DISCUSSION

IFN-β plays an important role in innate and adaptive immunity via IFN-stimulated genes containing IFN-stimulated response elements in their promoters (34). IFN-α/β has been shown to inhibit Chlamydia replication in vitro (5, 14, 16, 52), and therefore epithelial secretion of IFN-β may help establish a less permissive environment for Chlamydia replication early during infection. The dominant-negative TRIF cell line with the greatest inhibition of IFN-β secretion, (DN)TRIF1, had a significantly higher level of C. muridarum replication, possibly reflecting compromise of its innate response and/or resistance to infection.

In adaptive immunity, IFN-β contributes to dendritic cell maturation (19, 37), dendritic cell cross-priming of CD8 T-cell responses (32), IL-12 production (11), and Th1/Th2 polarization. IFN-β facilitates Th1 development in the presence of TNF-α (45). Mice deficient in IFN-β (39) or the IFN-α/β receptor (42) show a Th2 bias. IFN-β may contribute to the adaptive immune response against Chlamydia via dendritic cell activation and maturation, recruitment of lymphocytes through IFN-α/β-regulated chemokines, or direct effects on lymphocytes.

We and others have shown that Chlamydia species trigger IFN-β production in epithelial cells and macrophages (8, 13, 24, 31, 46). The identity of the PRR responsible for IFN-β secretion by Chlamydia-infected reproductive tract epithelial cells is not known. Our previous study utilizing oviduct epithelial cells identified TLR3, RIG-I, MDA5, or a putative cytosolic PRR that recognizes DNA. TLR3 is an endosomal PRR believed to play a role in surveillance for replicating RNA viruses. The only known microbial ligand for TLR3 is dsRNA (2). Similarly, RIG-I and MDA5 are cytosol resident PRRs that recognize dsRNA. It is not clear how these PRRs would recognize Chlamydia, which has no dsRNA structural subunit or replication intermediate. The putative cytosolic DNA PRR was potentially an attractive candidate PRR for Chlamydia induced IFN-β production by epithelial cells.

Based on the specific PRRs expressed by oviduct epithelial cells, identifying the signaling pathway leading to IFN-β would yield important information about the relevant upstream PRR. The identity of the PRR responsible for IFN-β secretion by Chlamydia-infected reproductive tract epithelial cells is not known. Our previous study utilizing oviduct epithelial cells identified TLR3, RIG-I, MDA5, or a putative cytosolic PRR that recognizes DNA. TLR3 is an endosomal PRR believed to play a role in surveillance for replicating RNA viruses. The only known microbial ligand for TLR3 is dsRNA (2). Similarly, RIG-I and MDA5 are cytosol resident PRRs that recognize dsRNA. It is not clear how these PRRs would recognize Chlamydia, which has no dsRNA structural subunit or replication intermediate. The putative cytosolic DNA PRR was potentially an attractive candidate PRR for Chlamydia induced IFN-β production by epithelial cells.
tor studies and siRNA experiments showed that *C. muridarum*-infected Bm1.11 epithelial cell production of IFN-β was dependent on viable *C. muridarum* and was largely TRIF-dependent and MyD88-independent. Partial knockdown of IRF3 with IRF3-specific siRNA supported the expected role for IRF3 in IFN-β production. We showed that cytosolic dsRNA [liposomal transfection of poly(I-C)] triggered IFN-β production through RIG-I/MDA5 in a TRIF-independent fashion (29, 43, 53, 55), a finding consistent with TRIF-independent signaling by RIG-I and MDA5 in murine oviduct epithelial cells (Fig. 6B). The dependence of *C. muridarum*-infected Bm1.11 epithelial IFN-β production on the TRIF adaptor rules out significant roles for RIG-I, MDA5, and the putative cytosolic DNA PRR in infection-triggered IFN-β production by Bm1.11 oviduct epithelial cells. TRIF dependence implicates TLR3 or an unknown TRIF-dependent PRR.

Our oviduct epithelial data complement the peritoneal macrophage data of Nagarajan et al. (46). IFN-β responses of oviduct epithelial cells and murine macrophages to *C. muridarum* infection differ in their dependence on MyD88, likely reflecting the differences in TLR molecules expressed by each cell type. *C. muridarum*-infected peritoneal macrophages from MyD88-deficient mice showed a 70 to 80% decrease in IFN-β mRNA compared to wild-type mice. Bafilomycin inhibition of endosomal acidification significantly blocked IFN-β mRNA induction in infected macrophages, implicating an endosomal TLR as the PRR responsible for IFN-β induction. TLR7 to TLR9 are localized within endosomes and require the MyD88 adaptor for IFN-β induction, leading us to postulate a major role for these TLRs in IFN-β production. Our oviduct epithelial cell lines do not express TLR7 to TLR9 by RT-PCR and do not respond to the TLR9 agonist ODN1826 (8). The existing literature supports TLR7 to TLR9 expression by macrophages, and peritoneal macrophages express TLR7 to TLR9 (data not shown), leading Nagarajan et al. (46) to postulate that infected primary murine lung fibroblasts from MyD88-deficient mice had decreased Cxcl10 (IP-10) production compared to wild-type controls. Cxcl10 transcription is upregulated by IFN-β and

**FIG. 6.** Bm1.11 oviduct epithelial cells respond to intracellular but not extracellular poly(I-C). (A) Supernatants were harvested from Bm1.11 cells that were treated with 50 or 100 µg of poly(I-C)/ml in epithelial cell medium, transfected with 50 µg of poly(I-C)/ml via Lipofectamine reagent in serum-free Dulbecco modified Eagle medium or Lipofectamine-only treated medium (Lipo only), or left untreated (Media). IFN-β secreted into the supernatants was measured at 24 h posttreatment. Pooled data from two independent experiments are shown. ND, not detected. (B) Bm1.11, (DN)TRIF1, (DN)TRIF2, and pcDNA3.1D vector control cells were treated with 75 µg of poly(I-C)/ml in serum (extracellular), transfected with 75 µg of poly(I-C)/ml via Lipofectamine reagent, treated with only Lipofectamine (Lipo only), or infected with 10 IFU of *C. muridarum* per cell. Supernatants were harvested 24 h after treatment, and the IFN-β levels were determined. Untreated Bm1.11 cells and dominant-negative cell lines were compared to the pcDNA3.1D vector control cells for each of the different treatment conditions. Pooled data from five independent experiments are shown. *, P < 0.05; ****, P < 0.0001; NS, not significant.

**FIG. 7.** Effect of blocking endosome acidification on *Chlamydia*-induced epithelial secretion of IL-6 and IFN-β. Epithelial cells were infected with 10 IFU of *C. muridarum*/cell. At 3 h postinfection, bafilomycin was added to the medium at the indicated concentrations. Supernatants were collected 24 h postinfection and analyzed for IFN-β (A) and IL-6 (B) by ELISA. (C) Cell monolayers were harvested at 30 h postinfection, and *C. muridarum* replication was quantified by determining the titers of sonicated suspensions on McCoy cells. Pooled data from two independent experiments are shown. IBs, inclusion bodies. *****, P < 0.0001.
can serve as an indirect marker for IFN-β production. Because fibroblast and epithelial cells are derived from different embryonic tissues (mesoderm versus ectoderm) and the TLR expression pattern of the lung fibroblasts was not characterized, it is not possible at this point to make direct comparisons of our oviduct epithelial cells and the primary lung fibroblasts with respect to IFN-β production. IFN-β production by murine peritoneal macrophages, bone marrow-derived macrophages (46), and oviduct epithelial cells (the present study) required viable \textit{C. muridarum}, suggesting that the relevant \textit{Chlamydia} ligand is either absent from the elementary body or inaccessible in that form. It may be that PRR triggering of IFN-β production requires \textit{Chlamydia} structural subunits (protein or otherwise) that are specific to the reticulate body or injection of \textit{Chlamydia} molecules into the cytosol via the putative type III secretion apparatus during replication.

Inhibition of endosomal acidification did not clarify a role for endosomal PRR recognition of \textit{C. muridarum} in infected Bm1.11 epithelial cells. Bafilomycin A had a major inhibitory effect on infected Bm1.11 cell secretion of IFN-β but had an even greater effect on \textit{C. muridarum} replication (Fig. 7). Nagarajan et al. reported that \textit{C. muridarum} replication was not significantly effected by bafilomycin A in peritoneal macrophages (46), \textit{Chlamydomonas} serovar L2 replicating in Vero cells is also indifferent to bafilomycin A (17). The sensitivity of \textit{C. muridarum} replication to bafilomycin A potentially identifies a pathogenicity factor for \textit{Chlamydia} replication in macrophages versus epithelial cells. Alternatively, the endosomal H⁺−proton pump inhibitor bafilomycin A is an antibiotic with known activity against gram-positive bacteria (62). Detailed studies of \textit{Chlamydia} susceptibility to bafilomycin have not been performed. It is possible that \textit{C. muridarum} has greater exposure to bafilomycin A within epithelial cells than it does within peritoneal macrophages.

Our data implicate TLR3, or an unknown TRIF-dependent PRR, as the PRR responsible for infected epithelial IFN-β production by \textit{C. muridarum}-infected oviduct epithelial cells. TLR3 is not a particularly appealing candidate PRR for IFN-β production since \textit{Chlamydia} lacks dsRNA structural subunits or replication intermediates and therefore has no obvious TLR3 ligand. It is possible that there are multiple alternative ligands for TLR3, as was the case for TLR2 (15, 35). Interestingly, Stockinger et al. (57) identified a TLR/Nod2-independent, IRF3-dependent pathway for IFN-β production in \textit{Listeria}-infected BMDM. These authors found that TRIF−/− bone marrow-derived macrophages (BMDM) had a marked decrease in IFN-β mRNA and Stat-1 phosphorylation (signaling event for IFN-α/β receptor) at 2 h postinfection but that IFN-β mRNA levels 6 h postinfection approximated those of the wild-type BMDM. The data were interpreted as showing a TRIF-independent pathway for IFN-β induction. It is unclear whether the delayed recovery of IFN-β at 6 h in \textit{Listeria}-infected TRIF−/− BMDM occurs via the same pathway responsible for induction of IFN-β at 2 h in wild-type BMDM. The delayed recovery of IFN-β could occur via a slower alternative pathway or via a redundant pathway triggered at a later point during the course of \textit{Listeria} infection. It is possible that the TRIF-dependent PRR IFN-β pathway utilized by \textit{C. muridarum}-infected Bm1.11 oviduct epithelial cells is the same TLR/Nod2-independent pathway identified in \textit{Listeria}-infected BMDM. The PRR for the TLR/Nod2-independent pathway in \textit{Listeria} infection has not been identified.

In summary, we showed that IFN-β secretion by \textit{C. muridarum}-infected oviduct epithelial cells required \textit{C. muridarum} replication and was dependent on IRF3 and TRIF. TLR expression analysis and TRIF dependence implicate TLR3 as the PRR responsible for infected epithelial IFN-β secretion. However, because TLR3 recognizes dsRNA, it would be an unexpected \textit{Chlamydia} PRR unless there is an uncharacterized non-dsRNA \textit{Chlamydia} TLR3 ligand. Neither inhibitor studies with bafilomycin (Fig. 7) nor TLR3 siRNA studies (data not shown) have generated definitive TLR3 data. Our bias is that there is a non-TLR3 TRIF-dependent pathway for IFN-β secretion in infected oviduct epithelial cells. Studies to definitively address this issue through derivation of TLR3−/− epithelial cell lines are under way in our laboratory.

**ACKNOWLEDGMENTS**

This project was supported by an NIAID K08 AI52128-01 grant to R.M.J. and NIH grants NIH ROI DE 13988-06 and NIH SPO1 AI56974-04 to S.C.H. W.A.D. was supported NIH training grants T32 AI07637 and T32 AI080519. We gratefully acknowledge the thoughtful comments of Stan Spinola and Byron Batteiger during evolution of the manuscript.

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