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Stimulator of IFN Gene Is Critical for Induction of IFN-β during Chlamydia muridarum Infection

Daniel Prantner,* Toni Darville,*†,1 and Uma M. Nagarajan*†

Type I IFN signaling has recently been shown to be detrimental to the host during infection with Chlamydia muridarum in both mouse lung and female genital tract. However, the pattern recognition receptor and the signaling pathways involved in chlamydial-induced IFN-β are unclear. Previous studies have demonstrated no role for TLR4 and a partial role for MyD88 in chlamydial-induced IFN-β. In this study, we demonstrate that mouse macrophages lacking TLR3, TRIF, TLR7, or TLR9 individually or both TLR4 and MyD88, still induce IFN-β equivalent to wild type controls, leading to the hypothesis that TLR-independent cytosolic pathogen receptor pathways are crucial for this response. Silencing nucleotide-binding oligomerization domain 1 in HeLa cells partially decreased chlamydial-induced IFN-β. Independently, small interfering RNA-mediated knockdown of the stimulator of IFN gene (STING) protein in HeLa cells and mouse oviduct epithelial cells significantly decreased IFN-β mRNA expression, suggesting a critical role for STING in chlamydial-induced IFN-β induction. Conversely, silencing of mitochondria-associated antiviral signaling proteins and the Rig-I–like receptors, RIG-I, and melanoma differentiation associated protein 5, had no effect. In addition, induction of IFN-β depended on the downstream transcription IFN regulatory factor 3, and on activation of NF-κB and MAPK p38. Finally, STING, an endoplasmic reticulum-resident protein, was found to localize in close proximity to the chlamydial inclusion membrane during infection. These results indicate that C. muridarum induces IFN-β via stimulation of nucleotide-binding oligomerization domain 1 pathway, and TLR- and Rig-I–like receptor-independent pathways that require STING, culminating in activation of IFN regulatory factor 3, NF-κB, and p38 MAPK. The Journal of Immunology, 2010, 184: 2551–2560.

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hlamydia trachomatis is the most common sexually transmitted bacterial infection in the world and can lead to pelvic inflammatory disease and infertility in women. In addition, ocular C. trachomatis serovars can cause trachoma, the leading cause of preventable blindness. It is well established that chlamydial infection of cells upregulates proinflammatory cytokines, chemokines, type I IFNs, and IFN stimulatory genes (1–4). Our recent discovery that type I IFN (IFN-α, IFN-β, interferon-stimulating factor) signaling is detrimental during the course of genital chlamydial infection in the mouse model (5) underscores the need to further elucidate the mechanism of production for these cytokines.

IFN-β is generated rapidly in response to viral and nonviral pathogens following recognition of conserved microbe-associated molecular patterns (MAMPs) by germline encoded pathogen recognition receptors (PRRs) (6). There are multiple parallel PRR pathways leading to IFN-β expression (7). Specifically, TLR3, TLR7, and the cytosolic Rig-I–like receptors (RLRs); RIG-I and melanoma differentiation associated protein 5 (MDA5) recognize viral RNA (8). In addition, DNA can be recognized by either TLR9 (9) in endosomes or cytosolic sensors, such as RNA polymerase III (10, 11) and the DNA-dependent activator of IFN regulatory factors (DAI) (12), in the cytoplasm. IFN-β–inducing MAMPs also include bacterial structures, such as Escherichia coli LPS recognized by TLR4 and cyclic di-GMP recognized by an uncharacterized cytosolic pathway (13).

The transmembrane and cytoplasmic PRR reside in different cellular compartments and use separate adaptor proteins for signal transduction. TLR3 and TLR4 use the adaptor molecule TRIF (14), whereas TLR7 and TLR9 use MyD88 (15, 16). Alternatively, nucleotide-binding oligomerization domain-like receptors (NLRs) require the host protein kinase Rip2 (17). The two RLRs, RIG-I, and MDA5, signal via the mitochondria located adaptor molecule mitochondrial antiviral signaling proteins (MAVS) (18), which is also known as VISA (19), IPS-1 (20) and CARDIF (21). Recently, the endoplasmic reticulum (ER) mitochondrial resident transmembrane protein stimulator of IFN gene (STING) (MITA/ERIS/MPYS/TMEM173) was discovered to function downstream of MAVS in the RLR pathway (22). Importantly, STING was also essential for IFN-β induction after recognition of cytosolic DNA, and can function independent of MAVS (23). A key aspect of all these PRR pathways is signal convergence by activation of a core set of transcription factors (NF-κB, AP-1, and IFN regulatory factor [IRF] family members), which are required for IFN-β expression (24).

With respect to chlamydial MAMPs, TLR signaling can be initiated by the binding of purified chlamydial LPS or heat shock protein 60 to TLR4 (25, 26), and the binding of a chlamydial lipoprotein to TLR2 (27). However, in the context of a chlamydial infection in vitro, TLR2 and not TLR4 seems to be a key component in chlamydial recognition to induce inflammatory cytokines (28).

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Abbreviations used in this paper: DKO, double knockout; ER, endoplasmic reticulum; IRF3, IFN regulatory factor; KO, knockout; MAMP, microbe-associated molecular patterns; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation associated protein 5; NOD, nucleotide-binding oligomerization domain; PDL, protein disulfide isomerase; poly I:C, polyinosinic acid-polycytidylic acid; PRR, pattern recognition receptors; RLR, Rig-I–like receptor; siRNA, small interfering RNA; STING, stimulator of IFN gene; UAMS, University of Arkansas for Medical Sciences; UI, uninfected cell; WT, wild type.

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In addition, nucleotide-binding oligomerization domain (NOD) 1, which recognizes bacterial cell wall motifs, plays a role in chlamydial-induced NF-κB activation in both human and mouse cells (29, 30). In regard to chlamydial-induced IFN-β induction, we have previously shown no role for TLR2 or TLR4, a partial role for MyD88, and a role for endosomal maturation (31), whereas another group has suggested a role for the adaptor molecule TRIF (32). Other than NOD1, the role of MyD88-independent cytosolic PRR pathways during chlamydial infection has not been properly examined. Although the two RNA helicases RIG-I and MDA5 are thought to be more important in viral recognition, RIG-I can also bind mRNA lacking a 5' cap (33, 34), such as bacterial RNA. Transfection of either bacterial mRNA (35), dsDNA (36), or cyclic di-GMP (13) potently activates the interferon response, suggesting that any of these pathways could be activated during chlamydial infection to induce IFN-β.

The goal of the current study was to characterize the MyD88-dependent and MyD88-independent signaling pathways used by the host cell to upregulate IFN-β during chlamydial infection. We found that individual contributions of TLRs that require MyD88 and endosomal maturation were dispensable for chlamydial-induced IFN-β. Similarly, TLRs signaling independent of MyD88 were dispensable, suggesting a contribution by non-TRL pathways in this response. Using RNA interference, we determined that chlamydial-induced IFN-β requires the adaptor molecule STING, but is independent of MAVS, RIG-I, and MDA5 in the RLR pathway. In addition, optimal IFN-β expression requires input from the NOD1 pathway. Furthermore, IFN-β expression is mediated by p38 MAPK and is completely dependent on IRF3 and partially dependent on IRF7.

Materials and Methods

Mouse strains

TLR4-/-MyD88 double knockout (DKO) mice were generated by crossing TLR4 knockout (KO; stock no. 007277, The Jackson Laboratory, Bar Harbor, ME) and MyD88 KO (37) (European Mouse Mutant Archive, Neuherberg/Munich, Germany) mice. IRF3 KO (stock no. RBRC008388) and IRF7 KO (stock no. RBRC01420) mice were purchased from RIKEN BioResource Center, Ibaraki, Japan. TLR7 KO mice (15) were a gift from Dr. Egil Lien (University of Massachusetts Medical School, Worcester, MA). TLR9 KO mice (9) were purchased from Oriental Yeast (Osaka, Ibaraki, Japan) IRF3 KO (38), IRF7 KO (39), TLR7 KO, and TLR4-/-MyD88 DKO mice used for experiments were bred in-house. Wild type (WT) control mice, TLR3 KO mice (B6; 129S1-Ticam1Lps2tm1Jgj, stock no. 005217), and TRIF-/- mice (C57BL/6-Ticam1tm10Jve/1, stock no. 000537) were purchased from The Jackson Laboratory. Control mice used for experiments were C57BL/6J for the IRF3 KO, IRF7 KO, TLR7 KO, TRIF KO, and TLR4-/-MyD88 DKO and B6129SF1/J for the TLR3 KO Committee. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Chlamydial stocks and cell lines

Chlamydia muridarum, Nigg strain, was propagated in Mycoplasma-free McCoy cells grown in DMEM media supplemented with 100 μM nonessential amino acids (Invitrogen), 2 mM l-glutamine (Invitrogen, Carlsbad, CA), 10% FBS, 50 mg/ml gentamicin sulfate, and 0.5 mg/ml cycloheximide. Infectious elementary bodies were isolated from McCoy cells by sonication, washed in PBS, resuspended in SPG buffer (250 mM sucrose, 10 mM sodium phosphate, and 5 mM l-glutamic acid, pH 7.2) and stored at −80°C. Oviduct epithelial cells (40) (B6; 11.1) were provided by Dr. Raymond Johnson (Indiana University, Indianapolis IN) and were cultured in Ham’s F12 media supplemented with 10% FBS, 12.5 ng/ml human recombinant Keratinocyte Growth Factor (Sigma-Aldrich, St. Louis, MO), 2 mM GlutaMAX (Invitrogen), and 50 μg/ml gentamicin. HEa cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM with 10% FBS, 1 mM l-glutamine, and 50 μg/ml gentamicin.

Chemicals and small interfering RNA reagents

E. coli LPS (100 μg/ml) was purchased from Sigma-Aldrich (cat. no. L2820) and E. coli or Toll-like receptor 3 (TLR3) - or other TLR or cytosolic PRR ligands poly inosinic acid:polycytidylic acid (poly I:C) (2.5 mg/ml), CpG DNA (10 μg), poly I:C/LyoVec (100 μg/ml), and poly dA:dT/LyoVec (100 μg/ml) was purchased from Sigma-Aldrich (cat. no. L2880) and poly I:C/LyoVec (10 μg/ml) was purchased from Pansorbin (Pansorbin, Winterthur, Switzerland) at a concentration of 50 μg/ml.

Macrophage infection

Peritoneal or bone marrow-derived macrophages were used for in vitro infections. Peritoneal macrophages were isolated 3 days after p.i. injection of 1 ml 5% thioglycollate into 8–10-week-old female C57BL/6J mice or KO mice and cultured in complete media (10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM l-glutamine, 100 μM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml β-mercaptoethanol). Bone marrow-derived macrophages were generated from flushing the femur bone marrow of 8–10-week-old female mice with RPMI. After 5 min incubation in an RBC lysis solution (17 mM Tris pH 7.4, 140 mM NaCl), and passage through a 70-μm nylon mesh filter, the cells were plated at a density of 4 × 10⁶ cells/ml in complete media containing 20 ng/ml recombinant murine M-CSF (Peprotech, Rocky Hill, NJ). M-CSF supplemented media was changed after days 2 and 4, and experiments were performed on days 6 or 7 in complete media lacking M-CSF.

In vitro infections and siRNA delivery

Infections of macrophages with C. muridarum were performed as previously described (31, 41). To confirm the cells were infected, macrophages in wells containing glass coverslips were fixed with methanol for 30 min at room temperature at 24 h postinfection and stained with the FITC conjugated polydinosamine anti-chlamydial mAb (Bio-Rad, Hercules, CA). Alternatively, infected macrophages were processed for IFU enumeration on a fresh McCoy monolayer (42). When the pharmacologic inhibitors U0126 (ERK1/2), SB203580 (p38), SP600125 (JNK), cytochalasin D and MG-132 were used, they were added to the cells 30 min before infection and replenished when the media was changed after the centrifugation step. For siRNA-mediated knockdown in cell lines, cells were first pretreated with the indicated siRNA or control duplexes for 56 h in Accell siRNA delivery media, which was then replaced with culture medium. Cells were infected with C. muridarum or treated with poly I/C or poly dA:dT 72 h after siRNA treatment. For all infections regardless of cell type, C. muridarum was added at one multiplicity of infection. At the indicated time points, supernatants were collected and stored at −80°C while the cell monolayers were processed for RNA extraction.

Cytokine analysis

The protein levels of IFN-β in culture supernatants were determined using an ELISA kit (BTL Biomedical Laboratories) following the manufacturer’s supplied protocol. Optical densities taken at 450 nm for quantification were measured using a Biotek plate reader.

RNA extraction and real-time PCR analysis

RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). RNA samples (1 μg) were treated with 1.0 U RNase-free DNase I (Promega, Madison, WI) for 30 min at 37°C followed by inactivation for 10 min at 70°C. Subsequently, 200 ng RNA was reverse transcribed with SuperScript III enzyme (Invitrogen) according to the manufacturer’s instructions, using random hexamer and oligo dt for priming. Quantitative PCR was performed on samples using an IQ-SYBR mix (Bio-Rad) and an iQ5 cycler (Bio-Rad). The amount of cDNA present was determined for each gene using the standard curve method and normalized to the housekeeping gene mouse β-actin or human GAPDH before analysis (31). All primers were designed using Beacon Design software (Bio-Rad) and given in Table 1.

Confocal microscopy

HEa cells (2 × 10⁵) were transfected according to the manufacturer’s instructions with 0.5 μg FLAG-tagged STING (Origene) using Optifect (Invitrogen). Cells were infected with one multiplicity of infection of C. muridarum 24 h after transfection as described previously. Cells were fixed at the indicated time points with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min, then permeabilized with PBS containing 0.2% Triton-X100 for 5 min. Cells were blocked in PBS containing 1% normal goat serum and stained using rabbit polyclonal anti-FLAG Ab (Cell Signaling Technology, Beverly, MA) at 1:300 dilution, followed by goat anti-rabbit Alexa Fluor 488 secondary Ab (Invitrogen) at 1:200 dilution. The coverslips were then mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen) and examined on a Zeiss LSM 710 confocal microscope.
mouse anti-PDI (Invitrogen) at 1:1000, or rabbit anti-sec61α (Abcam, Cambridge, MA) at 1:1000. Endogenous STING was stained using anti-MPSY (43) Ab (1:1000) provided by Dr. John Cambier. Mouse antisera against C. muridarum were used at 1:300 dilution. Alexa 488 conjugated anti-mouse and Alexa 568 anti-rabbit (Invitrogen) were used at 1:1000 dilution as secondary Abs for detection. Cells were washed and mounted using Prolong anti-fade containing DAPI (Invitrogen). Confocal images were acquired using Zeiss confocal microscope (LSM 510 META) and images analyzed using AxioVision software (Thornwood, NY).

Statistical analysis
When indicated, three independent experiments were performed to test for significance using SigmaStat (Systat, Evanston, IL). For analysis of cytokine gene expression, fold decreases were tested for significance by Student’s t test, with pairwise multiple comparison (Holm-Sidak method) was performed to determine statistically significant differences.

Results
Chlamydial-induced IFN-β occurs independently of TLRs
Although purified chlamydial LPS can signal via TLR4 (26), we have demonstrated that TLR4 is dispensable for IFN-β expression during chlamydial infection (28). It is possible that its role during infection is masked by compensation from other MyD88-dependent pathways, such as TLR2. To test this theory, macrophages from mice deficient in both the TLR4 ligand (Table I). TLR4-MyD88 DKO macrophages were unresponsive to E. coli LPS (1 μg/ml) or the TLR9 ligand CpG DNA (100 nM). Supernatants were assayed for IFN-β protein following 24 h of infection. Error bars in A and C represent the mean ± SD from three independent experiments.

FIGURE 1. IFN-β expression is unimpaired in TLR4-MyD88-DKO macrophages during chlamydial infection. IFN-β mRNA (A), IFN-β protein (B), and TNF-α mRNA (C) were quantified in WT and TLR4-MyD88 DKO peritoneal macrophages infected in vitro with C. muridarum and infected with either the TLR4 ligand LPS (1 μg/ml) or the TLR9 ligand CpG DNA (100 nM).
interferon response, IFN-β expression was almost completely lost in macrophages isolated from IRF3 KO mice (Fig. 4A). Conversely, IFN-β expression was only partially dependent on IRF7 (Fig. 4A, 4B). In macrophages, IRF3 is constitutively expressed whereas IFN-β is present only at low levels but can be induced by type I IFNs. Consequently, pretreatment of IRF3 KO macrophages with recombinant IFN-β restores the ability of IRF3 KO cells to induce IFN-β (Fig. 4C), correlating with an increase in IRF7 expression (data not shown). These findings indicate that initial IRF3-dependent IFN-β forms a positive feedback loop by inducing IRF7, which is then required for maximal IFN-β expression during chlamydial infection. In addition to activation of IRF3, NF-κB, and AP-1, family member activation is also necessary for IFN-β expression (46, 47). Treatment with a proteosomal inhibitor to block activation of NF-κB (48) totally abrogated the IFN-β response during infection of macrophages (Fig. 4D). ERK1/2, p38, and JNK MAPK are important for activation of AP-1 transcription factors (49). To determine which of these pathways was required for the IFN-β response during infection, macrophages were pretreated with pharmacologic inhibitors to individual members. Inhibition of p38 MAPK led to the greatest reproducible decrease (70–90% inhibition; p = 0.015) in IFN-β expression during infection (Fig. 4E), demonstrating that p38 is the most prominent MAPK activated by C. muridarum. The results from the above experiments suggest that the interferon response could occur independently of Toll-like receptors, culminating in activation of IRF3, NF-κB, and MAPK pathways. This finding leads to the conclusion that these pathways are likely triggered by cytosolic receptors following detection of chlamydial MAMPs.

**NOD1 is required for maximal IFN-β induction during chlamydial infection**

NOD proteins recognize structural motifs found in bacterial peptidoglycan and induce a MyD88-independent signaling pathway, activating NF-κB and leading to expression of proinflammatory cytokines (50). However, the contribution of NLRs in IFN-β induction during chlamydial infection has not been tested. The role of the NLR NOD1 during infection was elucidated by RNAi techniques in HeLa cells, as macrophages were difficult to transfect. Importantly, these cells express high levels of IFN-β mRNA following C. muridarum infection (Fig. 5A). Knockdown of NOD1 led to a 42–49% decrease in NOD1 mRNA in targeted cells (data not shown). This partial silencing was sufficient to reproducibly decrease (38–54% inhibition; p = 0.032) IFN-β expression in infected cells (Fig. 5A). Similarly, NOD1 knockdown also decreased (50–68% inhibition; p = 0.0068) the NF-κB-dependent gene IL-8 (Fig. 5B), indicating that the NOD1 pathway could be contributing to this response through activation of NF-κB, as previously reported (29, 30). The reduction in IFN-β mRNA following siRNA-mediated knockdown of NOD1 was not due to decreased bacterial growth or entry, as chlamydial 16S rRNA (rs16) levels remain unchanged (Fig. 5C).

**Chlamydia-induced IFN-β is dependent on STING, but not on the RLR adaptor MAVS**

Recently, a mitochondrial- and ER-located protein called STING was shown to be an important mediator of the type I IFN response downstream of multiple cytosolic pathways, but not TLRs (22, 23). The role of STING during infection was elucidated by RNAi techniques. STING knockdown in HeLa cells decreased (>80%) IFN-β upregulation following transfection of dsDNA (Fig. 6A), verifying the functional efficacy of silencing STING. Importantly, silencing of STING led to a significant decrease (70–85% inhibition; p = 0.0036) in IFN-β mRNA upregulation during infection with C. muridarum (Fig. 6B), implying that STING is a crucial component of this response. Furthermore, the observation that chlamydial rs16 was not reduced as a result of STING knockdown (Fig. 6D) strongly indicates that the difference in the interferon response owing to STING knockdown is not mediated
by growth restriction of C. muridarum. Importantly, STING knockdown did not affect IL-8 mRNA levels in infected HeLa cells (Fig. 6C), suggesting that STING is not required for NF-κB activation during chlamydial infection.

The mitochondrial protein MAVS is a common adaptor molecule in the RLR pathway functioning upstream of STING. MAVS is essential for relaying the signals from the RNA helicases RIG-I and MDA5 to induce type I IFNs during viral infection or following cytosolic delivery of dsRNA (18–21, 51, 52). Knockdown of MAVS led to decreased IFN-β induction (60–70% inhibition) following treatment with transfected poly I:C (Fig. 6E). Conversely, knockdown of MAVS had no effect on expression of IFN-β following chlamydial infection (Fig. 6F). Likewise, siRNA mediated knockdown of RIG-I, and MDA5 had no effect on chlamydial-induced IFN-β (data not shown). These results suggest activation of STING during C. muridarum infection occurs independently of MAVS and RNA helicases.

To further establish whether STING is necessary for chlamydial-induced IFN-β in mouse cells derived from the female genital tract, primary mouse oviduct epithelial cells (Bm1.11) were also examined. STING knockdown in these cells led to decreased upregulation of IFN-β (91–95% inhibition; \( p = 0.016; \) Fig. 7A) and the IFN-β inducible protein CXCL10 (72–81% inhibition; \( p = 0.038; \) Fig. 7B) during infection. Chlamydial rs16 expression was independent of STING silencing (Fig. 7C), indicating that the impairment in IFN-β induction following STING knockdown was not caused by limiting chlamydial development. Cumulatively, these results demonstrate that chlamydial-dependent activation of the host interferon response in mouse and human cells require the host protein STING.

Intracellular trafficking of STING during chlamydial infection

Prior work has demonstrated that STING is basally located in the mitochondria and the ER. In light of its important role in chlamydial-induced IFN-β upregulation, it was of interest to determine whether there was an interaction between STING and the chlamydial inclusion. Trafficking and localization of this protein during infection was initially examined using HeLa cells transfected with FLAG-tagged STING. In the absence of infection, STING was found to colocalize with the ER marker protein disulfide isomerase (PDI), consistent with its ER-localization. Interestingly, chlamydial infection led to redistribution of STING staining surrounding the inclusion (Fig. 8A). The ER marker PDI also colocalized with FLAG-STING on the inclusion membrane (Fig. 8B). To rule out the possibility that the observed localization of FLAG-STING was not a result of overexpression, localization of endogenous STING was...
determined using anti-MPYS (STING) Ab. Again, a distinct staining of endogenous STING was observed around the inclusion (Fig. 8C). Whether the staining of STING reflects recruitment to the inclusion membrane is unknown. However, colocalization of ER markers PDI and Sec 61α to the inclusion membrane (Fig. 8D) suggested that either the ER may be in close proximity to the inclusion membrane or vesicular fusion of ER with the inclusion membrane occurred. The colocalization of Sec61α to the inclusion membrane is particularly notable, as STING has been demonstrated previously to associate with the translocon protein Sec61β (23). The close proximity of STING to the inclusion membrane suggests that its localization could be critical for IFN-β upregulation (Fig. 9).

Discussion
Type I IFNs are known for their antiviral activity. Conversely, they have been demonstrated to be detrimental during infection with C. muridarum in the lung (53) and genital tract (5) and also during systemic Listeria monocytogenes infection (54). However, the mechanistic basis underlying the production of IFN-β remains largely unknown for Chlamydia spp. and many other nonviral pathogens. This study was undertaken in an attempt to better characterize this process. Previously, the TLR2-MyD88 pathway was found to be important for upregulation of many proinflammatory cytokines, such as TNF-α and IL-6, during chlamydial infection of macrophages in vitro (28). However, it was also demonstrated that TLR2 was not essential for expression of IFN-β and interferon response genes in this same cell type (31). This finding illustrates a fundamental point that the cumulative host response during infection is controlled by multiple PRRs signaling pathways inside the cell.

Our aim was to examine these TLR2-independent pathways and how they contribute to the chlamydial-induced IFN-β response. Overall, the fact that expression of IFN-β was unimpaired in TLR4-MyD88 DKO, TLR3 KO, TLR7 KO, TLR9 KO, and TRIFKO cells during infection strongly indicated that chlamydial-induced IFN-β expression occurs mostly independent of TLRs. However, it must be noted that this conclusion conflicts with a previous report that IFN-β mRNA upregulation in an oviduct epithelial cell line (40) infected with C. muridarum was partially dependent on the TRIF pathway (32). This finding was illustrated using a combination of siRNA techniques and a dominant negative TRIF construct. However, this latter construct consisting of only a truncated TIR domain has been shown to have nonspecific effects.
on multiple non-TRIF pathways (55). In addition, we previously observed a partial inhibition of IFN-β in MyD88 KO cells (31). However, this inhibition was lost in TLR4-MyD88 DKO macrophages, suggesting that TLRs could still have a regulatory role in IFN-β induction during chlamydial infection. Interestingly, TLR-independent IFN-β upregulation is consistent with separate studies analyzing the interferon response during infection with many other nonviral pathogens, such as *Listeria monocytogenes* (56), *Legionella pneumophila* (36), *Helicobacter pylori* (35), *Francisella novicida* (57), Group B streptococci (58), and *Trypanosoma cruzi* (59).

This is especially noteworthy considering that these pathogens exhibit extremely diverse development cycles.

An attractive possibility for recognition of intracellular prokaryotic pathogens is by the NLR proteins, which recognize structural elements found in bacterial cell walls. NOD1, which specifically recognizes γ-D-glutamyl-meso-diaminopimelic acid (60), has been found to contribute to NF-κB activation (29) and IL-8 expression (30) during chlamydial infection. However, NOD1 signals via RIP2, which has been demonstrated to activate NF-κB (50) and AP-1 transcription factors (61), but not IRF family members. Previous work in HeLa cells demonstrated a role for NOD1 in IL-8 expression during chlamydial infection (30). Similarly, our results showed that knockdown of NOD1 led to a consistent decrease in chlamydial-induced IFN-β in HeLa cells, suggesting that NOD1 could be contributing toward optimal NF-κB activation required for IFN-β induction. Furthermore, there must be another PRR working in tandem with NOD1 that contributes to activation of IRF transcription factors. In this regard, it is important to note that NOD2, another NLR that can activate IRF3 via MAVS to induce IFN-β during infection with respiratory syncytial virus (62), is not expressed in HeLa cells.

A recent study demonstrated that the IFN-β response to *L. monocytogenes* was lost in STING KO mouse embryonic fibroblasts (23), hinting that this newly characterized protein might be a major component of the TLR-independent interferon response. In agreement with this study, we have shown by siRNA silencing techniques that STING is also an important mediator of IFN-β induced by *C. muridarum* in both mouse oviduct epithelial cells and human cells, suggesting that irrespective of cell type, STING plays a central role in Chlamydial-induced IFN-β and the subsequent expression of interferon response genes. STING has been shown to function downstream of the common adaptor molecule MAVS (22, 23).

**FIGURE 8.** STING colocalizes with the ER and accumulates in the vicinity of the inclusion during infection. HeLa cells were transfected with a plasmid vector expressing human flag tagged STING and infected for 20 h with *C. muridarum*. A, Single color confocal microscopy staining for STING (red), *C. muridarum* (green), nuclei (blue), and a merged picture. B, Single color staining for STING (red), ER resident protein PDI (green), nuclei (blue), and a merged picture. Arrows indicate chlamydial inclusions. C and D, Staining for endogenous STING and Sec61α (red) respectively, chlamydial inclusion (green), nuclei (blue), and merged pictures. UI, uninfected cells.

**FIGURE 9.** Schematic representation of pathways contributing to IFN-β induction during chlamydial infection.
23). However, knockdown of the MAVS or the upstream RLR pathway components MDA5 and RIG-I did not impair the interferon response during infection with C. muridarum. Similarly, the response to L. monocytogenes also proceeds independently of MAVS (63), leading to the pertinent question of what cytosolic pathway is upstream of STING. Cytosolic delivery of dsDNA or infection with a DNA virus, such as HSV I, can lead to the type I IFN response in a STING-dependent, but RIG-I–independent manner (22). Therefore, it is theoretically possible that a DNA sensor could be the common upstream receptor protein. Known DNA sensors include the host proteins DAI (12), absent in melanoma 2 (64), and RNA polymerase III (10, 11). However the role of DAI has been suggested to be unimportant for IFN-β expression in many cell types treated with dsDNA (65), absent in melanoma 2 has been limited to activation of caspase-1 and not IFN-β (64) and RNA polymerase III recognizes only poly(dA-dT). Interestingly, using chimeric constructs it was determined that dimerization of STING was sufficient to induce signaling (66). Therefore, it is also conceivable that accumulation of STING around the chlamydial inclusion membrane could facilitate STING dimerization and potentially bypass the need for an upstream receptor protein. STING was found to basally reside in the ER as previously reported (22, 23), but postinfection STING also appears to localize on the inclusion membrane. No enrichment of STING was observed in the mitochondria following infection, consistent with the lack of the role of mitochondrial MAVS in chlamydial-induced IFN-β. Based on staining of other ER markers, it is possible that the ER is present in close proximity to the inclusion membrane (Dr. P. B. Wyrick, personal communication). Alternatively, chlamydial Ags, such as MOMP and LPS, localize to the ER during infection (67), suggesting that vesicular fusion between ER and inclusion membrane might be taking place. Regardless of these two possibilities, at this point it is unclear whether the trafficking of STING to the vicinity of the inclusion is absolutely necessary for its ability to signal during infection.

We have demonstrated that chlamydial-induced IFN-β expression during infection is completely dependent on IRF3 and partially dependent on IRF7 (Fig. 4). STING has been shown to interact with IRF3 (22), suggesting it to be a major player in IRF3 activation. Pretreatment with recombinant IFN-β rescued the ability of IRF3 KO macrophages to upregulate IFN-β, supporting the notion that basal levels of IRF7 are too low to compensate for the IRF3 deficiency in these cells at restating. In addition to IRF transcription factors, other cellular pathways leading to IFN-β upregulation included p38 MAPK and NF-kB activation. The INK MAPK inhibitor also led to a slight but reproducible decrease in IFN-β expression in macrophages. Numerous studies have demonstrated that MAPK can phosphorylate and activate AP-1 transcription factors (49). Activation of IRF3 and NF-kB can be mediated downstream of PRRs, but p38 MAPK also regulates NF-kB activation (68), while INK can activate IRF3 by directly phosphorylating serine 173 residue (69). Clearly, several pathways have to function to induce IFN-β, with IRF3 activation being critical. Based on our results, it can be speculated that the decrease in IFN-β expression during infection of NOD1 silenced cells is a result of decreased NF-kB and AP-1 activation (Fig. 9). This speculation is supported by the findings that STING knockdown did not affect IL-8 expression, indicating that STING is not important for NF-kB activation during chlamydial infection. Conversely, STING knockdown is detrimental to IFN-β induction, because it is the only pathway available to activate IRF3 and IRF7 in HeLa cells.

Our data present strong evidence that the host protein STING plays a key role in IFN-β expression in cells infected with C. muridarum by mediating activation of IRF3. Importantly, this identifies a novel arm of the host innate immune response activated during chlamydial infection. Because induction of IFN-β during chlamydial infection is detrimental to the host, it is an interesting possibility that intracellular bacteria are modulating the viral IFN-β induction pathways for their own benefit. The nature of the chlamydial MAMPs inducing the IFN-β response and how they are delivered to the cytosolic detection system are future questions to be addressed. As noted, the possible MAMPs include, but are not necessarily limited to, pathogen DNA or cyclic di-GMP. Numerous studies have linked IFN-β expression to bacterial secretion systems (36, 70–72), including the type III secretion apparatus in C. muridarum (41), which could be delivering these MAMPs to cytosolic PRRs. The next step will be to determine the mechanism by which STING recognizes the IFN-β–inducing MAMP during chlamydial infection.

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Disclosures

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