Role of STAT1 in *Chlamydia*-Induced Type-1 Interferon Production in Oviduct Epithelial Cells

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We previously reported that *Chlamydia muridarum*-infected murine oviduct epithelial cells (OE cells) secrete interferon β (IFN- β) in a mostly TLR3-dependent manner. However, *C. muridarum*-infected TLR3-deficient OE cells were still able to secrete detectable levels of IFN- β into the supernatants, suggesting that other signaling pathways contribute to *Chlamydia*-induced IFN- β synthesis in these cells. We investigated the role of STAT1 as a possible contributor in the *Chlamydia*-induced type-1 IFN production in wild-type (WT) and TLR3-deficient OE cells to ascertain its putative role at early- and late-times during *Chlamydia* infection. Our data show that *C. muridarum* infection significantly increased STAT1 gene expression and protein activation in WT OE cells; however, TLR3-deficient OE cells showed diminished STAT1 protein activation and gene expression. There was significantly less IFN- β detected in the supernatants of *C. muridarum*-infected OE cells derived from mice deficient in STAT1 when compared with WT OE cells, which suggest that STAT1 is required for the optimal synthesis of IFN- β during infection. Real-time quantitative polymerase chain reaction analyses of signaling components of the type-1 IFN signaling pathway demonstrated equal upregulation in the expression of STAT2 and IRF7 genes in the WT and TLR3-deficient OE cells, but no upregulation in these genes in the STAT1-deficient OE cells. Finally, experiments in which INFAR1 was blocked with neutralizing antibody revealed that IFNAR1-mediated signaling was critical to the *Chlamydia*-induced upregulation in IFN- α gene transcription, but had no role in the *Chlamydia*-induced upregulation in IFN- β gene transcription.

Introduction

CHlamydia trachomatis is the most common bacterial sexually transmitted disease in the United States (Miller and others 2004). Since *C. trachomatis* infections are often asymptomatic, the absence of clinical symptoms greatly contributes to the spread of *Chlamydia* to uninfected individuals, which makes *C. trachomatis* infection a major public health concern (Tilson and others 2004). Chronic infections with urogenital serovars of *C. trachomatis* and ascension of *Chlamydia* into the upper reproductive tract epithelium can cause pelvic inflammatory disease, scarring, and infertility (Moller and others 1979; Chow and others 1990).

Epithelial cells lining the genital tract act as sentinels for invading *Chlamydia*. These cells produce and secrete a multitude of cytokines and chemokines in response to chlamydial infection, which mediate pathogen restriction and recruitment of adaptive immune cells (T-cells) to the site of infection (Kagnoff and Eckmann 1997; Rasmussen and others 1997). In mammalian cells, invasion by microorganisms is sensed via a specialized family of membrane-bound pattern recognition receptors (PRRs) called Toll-like

receptors (TLRs) (Takeda and others 2003). The TLRs are stimulated by the recognition of pathogen-associated molecular patterns (PAMPs) and trigger synthesis of acute inflammatory cytokines and chemokines including Rantes (CCL-5), IL-6, GM-CSF, IL-1 β , and type-1 interferon (IFN) (Rasmussen and others 1997; Takeda and others 2003; Netea and others 2004; Rank and others 2010). Because the immune response to *Chlamydia* infection of epithelial cells results in the TLR-dependent synthesis of immune factors that are directly and/or indirectly involved in leukocyte recruitment, activation, and polarization (Johnson 2004; Derbigny and others 2005; Commins and others 2010), TLRs are now considered critical components in the primary innate inflammatory response to *Chlamydia* infection, and in the initiation of adaptive immune responses.

We previously showed that *Chlamydia muridarum* infection of murine oviduct epithelial (OE) cells induces TLR-dependent synthesis of IL-6, GM-CSF, and IFN- β (Derbigny and others 2005, 2012). Subsequent studies identified TLR3 as the primary PRR stimulated in OE cells, which mediates the *Chlamydia*-induced synthesis of IFN- β in a TRIF-dependent manner (Derbigny and others 2005, 2007, 2010).

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However, our data also revealed that the OE cells still produce residual amounts of IFN- β in the absence of functional TLR3. The ability of the OE cells to secrete residual amounts of *Chlamydia*-induced IFN- β in the absence of TLR3 implicates the existence of additional pathways that may be involved in this response. Other studies have shown that *Chlamydia* can induce type-1 IFN synthesis via multiple routes including the MYD88-dependent pathway in peritoneal macrophages (Nagarajan and others 2005), through the RAS signaling adaptor molecule cPLA2 in mouse embryo fibroblasts (Vignola and others 2010), and by TLR-independent pathways mediated by nucleotide-binding oligomerization domain 1 (NOD1) and stimulator of IFN gene (STING) protein (Prantner and others 2010). An investigation into the role of STAT1 in response to *Chlamydia*-induced type-1 IFN synthesis in HeLa 229 cells demonstrated a robust IFN- β -dependent upregulation and increased activation of components of the JAK/STAT signaling pathway that was essential for restricting pathogen replication (Lad and others 2005). The existence of multiple pathways by which *Chlamydia* can induce type-1 IFN production emphasizes redundancy in immune responses to infection, and highlights the importance of type-1 IFNs in *Chlamydia* pathogenesis.

We investigated the role of STAT1 and JAK/STAT signaling in the *Chlamydia*-induced IFN- β response in OE cells, to ascertain whether JAK/STAT signaling pathways contribute to IFN- β synthesis in OE cells as was implied in the studies using HeLa 229 cells. Based on observations that *C. muridarum*-induced IFN- β synthesis is TLR3-dependent in OE cells, we hypothesize that STAT1 is activated subsequent to the TLR3-dependent response in OE cells to sustain and amplify the type-1 IFN responses during infection. We also hypothesized that JAK/STAT signaling is a compensatory mechanism for *Chlamydia*-induced IFN- β production in the absence of TLR3, and wanted to ascertain whether this pathway was indeed more active in type-1 IFN synthesis in TLR3-deficient OE cells.

Our results show that STAT1 expression and activation is upregulated in response to *C. muridarum* infection in OE cells, and that STAT1 is critical for IFN- β production late in infection. We demonstrate that TLR3-deficiency diminishes expression and activation of STAT1 in infected OE cells late in infection, suggesting that optimal levels of *Chlamydia*-induced IFN- β is dependent on initial TLR3-dependent production of IFN- β . We also demonstrate that STAT1 differentially modulates *Chlamydia*-induced expression of key type-1 IFN signaling pathway components during early and/or late stages of infection. In this study, we present data that suggest STAT1 plays a critical role in modulating the innate immune response in OE cells at late times during *Chlamydia* infection, by amplifying type-1 IFN signaling and production.

Methods

Mice and ethics statement

C57BL/6J (control) and C57B6-*stat1*^{-/-} (STAT1-deficient) matched female mice were a gift from Dr. Akira Moh and Dr. Xin-Yuan Fu at Indiana University School of Medicine, Department of Microbiology and Immunology. The mice used in these experiments were developed in-house by Dr. Xin-Yuan Fu, and the mice were immediately sacrificed so that OE

cell lines could be derived for these and for future studies. The Indiana University Institutional Animal Care and Use Committee (IACUC) approved all experimental animal protocols. All care was used to ensure that steps were taken to ameliorate animal suffering in all work involved in the removal of oviduct tissue.

Reagents

Recombinant murine IFN- γ was purchased from Pepro-Tech (Rocky Hill, NJ). The lyophilized IFN- γ was suspended in phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin and frozen at -20°C until use. Immediately before use, recombinant murine IFN- γ was thawed and diluted to 10 ng/mL in fresh epithelial cell media: 1:1 Dulbecco's modified Eagle's medium:F12K (Sigma-Aldrich, St. Louis, MO), supplemented with 10% characterized fetal bovine serum (Thermo-Fisher, Pittsburgh, PA), 2 mM L-alanyl-L-glutamine (GlutaMAX I; Life Technologies/Invitrogen, Carlsbad, CA), 5 mg bovine insulin/mL, and 12.5 ng/mL recombinant human fibroblast growth factor-7 (keratinocyte growth factor; Sigma-Aldrich). Recombinant murine IFN- β was purchased from R&D Systems (Minneapolis, MN). Recombinant murine IFN- β was reconstituted, stored, and used as previously described (Derbigny and others 2012). Purified anti-mouse type-1 interferon- α/β receptor1 (IFNAR1) neutralizing antibody against subunit 1 of the heterodimeric type-1 IFN receptor, and the mouse IgG1 isotype control were purchased from Biolegend (San Diego, CA). The neutralizing antibody and isotype control were stored according to the manufacturers' instructions, and diluted in fresh epithelial cell media for neutralization experiments.

Cells and bacteria

Derivation and maintenance of cloned OE cell lines OE129 TLR3 (-/-) and OE129 WT were done as previously described (Johnson 2004; Derbigny and others 2005, 2010), and the OE cell lines OEB6 STAT1 (-/-) and OEB6 WT (described for the first time here) were derived using the identical methodology used to derive the OE129 cell lines. Briefly described, reproductive tract tissues from C57BL/6J (control) and C57B6-*stat1*^{-/-} (STAT1-deficient) female mice, encompassing a small cuff of the ovary, were harvested. Luminal epithelial cells were released with a pancreatin-hyaluronidase-collagenase mixture. The resulting epithelial cells were expanded *in vitro* and cloned by limiting dilution. Resulting clones were confirmed for epithelial origin by screening for IFN- γ -inducible MHC class II as previously described (Johnson 2004; Derbigny and others 2005, 2010). Selected clones were expanded and designated OE STAT1 (-/-) and OEB6 WT from STAT1-deficient and wild-type (WT), respectively. These cells were grown at 37°C in a 5% CO₂ humidified incubator and maintained in epithelial cell media as previously described (Johnson 2004; Derbigny and others 2005, 2007).

Mycoplasma-free *C. muridarum* was grown and titered in McCoy cells (American Type Culture Collection), as previously described (Schachter and Caldwell 1980; Johnson 2004).

Infections

OE129 WT, OE129 TLR3(-/-), OEB6 WT, and OE STAT1(-/-) cells were plated in 24-well tissue culture

plates and used when 80%–90% confluent. For all experiments, the cells were infected with 10 inclusion-forming units (IFU) of *C. muridarum*/cell in 750 μ L of epithelial cell culture medium. The plates were centrifuged at 1,200 rpm ($250\times g$) in a tabletop centrifuge for 1 h then incubated at 37°C in a 5% CO₂ humidified incubator without subsequent change of medium for 24 h. Mock-infected, cytokine-only treated, and antibody-only treated wells received an equivalent volume of epithelial cell culture medium lacking *C. muridarum*.

Analysis of chlamydial growth in OE cells

OEB6 WT and OE STAT1 (–/–) cells were plated in 24-well tissue culture plates and either mock infected, or infected with 5 IFU of *C. muridarum*/mL as described above. At 30 h postinfection, the cell lysates containing *Chlamydia* were harvested by mechanically scraping with a pipette tip in 500 μ L of SPG buffer and frozen at –70°C until further processed. To study infectivity, the collected infected cell lysate samples were vortexed and sonicated for 15 min in a water bath, and 50 μ L of the sample was passaged onto a fresh layer of McCoy cells for titering as described above.

ELISA determination of cytokine production

OE129 WT, OE129 TLR3 (–/–), OEB6 WT, and OE STAT1 (–/–), were plated in 24-well tissue culture plates and were grown to 80%–90% confluency. The cells were either infected with 10 IFU of *C. muridarum*/cell and/or treated with the appropriate cytokine or antibody at the concentrations specified in the text. Supernatants were harvested at either 24 h post-infection or the indicated time points, and analyzed for cytokine content using custom ELISA for IFN- β as previously described (Derbigny and others 2005). All standards and experimental samples were analyzed in triplicate. The lower range of assay sensitivity for IFN- β was 10 pg/mL. Optical densities taken at 450 nm for quantification were measured using a microplate reader (Bio-Rad, Hercules, CA).

Western blotting

OE129 WT, OE129 TLR3 (–/–), OEB6 WT, and OE STAT1 (–/–) cells were plated in 24-well tissue culture plates and grown to 80%–90% confluency. Subsequently, these cells were either mock-infected, IFN- γ -treated, or *C. muridarum*-infected for indicated time points. After removal of the cell supernatants, the cells were gently washed with PBS. Soluble proteins were then extracted with a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS purchased from Imgenex (San Diego, CA), and a mixture of protease inhibitor cocktail at 1 μ L/mL (Sigma); while incubated on ice for 30 min. Cell lysates were clarified by centrifugation at 14,000 rpm ($20,000\times g$), and proteins were quantified and separated by SDS-PAGE as previously described (Derbigny and others 2007). After proteins were transferred to nitrocellulose transfer membranes (Bio-Rad), the transfer membranes were blocked according to manufacturer's protocol with a protein-free Tris-buffered saline (pH 7.4) containing 0.05% Tween-20 blocking buffer purchased from Thermo-scientific, Pierce (Rockland, IL). The proteins were stained by immunoblotting with either a 1:2,000 dilution of a murine antibody

against total STAT1, or a 1:2,500 dilution of phosphorylation-specific (pY701) STAT1 antibody (both obtained from BD Bioscience San Jose, CA). Protein-antibody complexes were detected by secondary blotting with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (Thermo scientific, Pierce). Proteins were visualized using the ECL western blotting substrate (Thermo scientific, Pierce) as described in the manufacturer's protocol.

Recombinant cytokine treatment

To serve as a positive control for the induction of STAT1 protein activation and STAT1 protein/gene expression, recombinant murine IFN- γ was added directly to the media of OE129 WT, OE129 TLR3(–/–), OEB6 WT, and OE STAT1(–/–) cells at 10 ng/mL for 4–6 h before being harvested or mock-infected with epithelial cell medium lacking viable *Chlamydia*. Recombinant IFN- β treatment was achieved by adding recombinant IFN- β directly to the medium of the cells at 50 U/mL, and the cells were allowed to incubate for an additional 4 h before cells were harvested and analyzed for induction of genes involved in the autocrine/paracrine pathways for IFN- β synthesis. To simulate autocrine/paracrine events of an “early” *Chlamydia* infection, 50 U/mL of the recombinant IFN- β was added to the OE cells 2 h after being mock-infected with epithelial cell medium lacking viable *Chlamydia*, and the cells were harvested after an additional 4 h (6 h PI). To mimic autocrine/paracrine events of “late” *Chlamydia* infections, 50 U/mL recombinant IFN- β was added to the OE cells 2 h after being mock-infected with epithelial cell medium lacking viable *Chlamydia*, allowed to incubate with the cells for 4 h to represent the IFN- β being secreted early during normal *Chlamydia* infection, and the media was replaced with fresh media at 6 h PI. An additional 50 U/mL aliquot of recombinant IFN- β was added to the OE cells at 12 h postinfection, and the cells were harvested after incubation at 37°C for an additional 4 h (16 h PI).

Neutralization experiments

For IFNAR neutralization experiments, the OE cells were either infected with *C. muridarum* or treated with IFN- β , while IFNAR signaling was blocked with a neutralizing antibody against murine IFNAR1 at either early-stage (6 h) or late-stage *C. muridarum* infection (16 h). For 6 h time points, OE cells were either infected with 10 IFU/cell *C. muridarum* and 1 μ g/mL IFNAR1 neutralizing antibody added to the cell supernatants at 1 h postinfection, or had 1 μ g/mL IFNAR1 neutralizing antibody added to the cells 1 h after being mock-infected with epithelial cell medium lacking viable *Chlamydia* (recombinant IFN- β experiments). For 16 h time points, either OE cells were infected with 10 IFU/cell *C. muridarum* and 1 μ g/mL IFNAR1 neutralizing antibody was added directly to the cell supernatants at 11 h post-infection, or 1 μ g/mL IFNAR1 neutralizing antibody was added to the cells 11 h after being mock-infected with epithelial cell medium lacking viable *Chlamydia* in the recombinant IFN- β experiments. The cells were harvested for real-time quantitative polymerase chain reaction (RT-qPCR) analyses at the conclusion of either the 6 or 16 h time-points. Normal mouse IgG1 isotype antibody was used in simultaneously conducted control experiments.

RT-PCR and real-time quantitative PCR

Total cell mRNA was isolated from mock-infected, IFN- γ -treated, and *C. muridarum*-infected OE129 WT, OE129 TLR3 (–/–), OEB6 WT, and OE STAT1 (–/–) cells using the RNeasy kit (Qiagen, Valencia, CA). All samples were treated with RNase-free DNase I (Qiagen) to remove genomic-DNA and were quantified by spectrophotometric analysis. One to 2 μ g of total RNA were then reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's protocol.

RT-PCR. Optimized primer pairs for STAT1 and β -actin (Table 1) were designed by using the Primer 3 design tool (Rozen 2000), and 2 μ L of cDNA template was amplified by PCR using the following cycling conditions: 2 min of initial denaturation at 95°C; followed by 35 cycles of 30 s at 95°C, 15 s at 60°C, and 30 s at 68°C. During the 35th cycle, the 72°C extension was 2 min to complete the PCR. Reactions were also amplified in the absence of reverse transcriptase as negative controls.

RT-qPCR. total RNA was purified from OE cells using the RNeasy Plus kit (Qiagen) as described above. One microgram of total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. Primer pairs were designed from published data and databases (Cui and others 2007; Roth-Cross and others 2007; Table 1), and were adjusted to 2 pmol/ μ L working stock. RT-qPCR was conducted with the diluted cDNA and primers as per the protocol outlined in the iScript One-Step RT-PCR with SYBR Green kit (Bio-Rad), in the ABI 7000 thermal cycler (Bio-Rad). Dissociation curves were recorded after each run to ensure primer specificity. Relative expression was calculated using $2^{-\Delta\Delta CT}$ method as previously described (Livak and Schmittgen 2001).

Immunofluorescent staining

OEB6 WT, and OE STAT1 (–/–) cells were cultured in a 24-well tissue culture plate and allowed to grow to 70% confluence. The cells were then mock-infected or infected with *C. muridarum* as previously described. At 24 h post-infection, the infected cells were then fixed with 200 μ L of methanol and incubated at room temperature for 10 min. The fixed cells were stained with a murine anti-*Chlamydia* LPS antibody, provided by Dr. Wilbert J. Newhall (Jones and others 1986), diluted 1:500 in PBS, and incubated for 1 h at room temperature. The stained cells were washed 3 times with PBS. Detection was accomplished with a secondary stain of fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG antibody (Rockland Immunochemicals, Gilbertsville, PA) diluted 1:50 in PBS.

Statistical analysis

Data are expressed as mean $\pm$ SD. All experiments were repeated at least 3 times, and statistical significance determined using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

C. muridarum induces STAT1 protein activation/ expression and STAT1 gene expression

To identify a role for STAT1 in the context of type-1 IFN synthesis and signaling in *C. muridarum*-infected OE cells, we examined STAT1 gene expression and protein activation in WT OE cells via RT-PCR and western blot analysis, respectively. OE cell lines derived from WT 129S1 mice (OE129 WT) and WT C57BL/6 mice (OEB6 WT) were either mock-infected, treated with IFN- γ (10 ng/mL), or infected with 10 IFU/cell of *C. muridarum*. Figure 1 shows representative data of OE cells harvested at 24 h PI.

As shown in Fig. 1A, *C. muridarum* infection increased STAT1 mRNA levels in both WT OE cell lines to a level comparable to that of the IFN- γ -treated controls (Darnell 1997; Levy and Darnell 2002). Expression of total STAT1 protein and activated STAT1 (phosphorylated STAT1) was also higher in *C. muridarum*-infected WT OE cells versus mock-infected control cells (Fig. 1B). These increases were comparable to those observed in IFN- γ -treated controls (Fig. 1C). Collectively, the data indicate that *C. muridarum* induces STAT1 expression and activation in OE cells similarly as was observed in *C. trachomatis* infected HeLa 229 cells (Lad and others 2005).

STAT1 is required for *C. muridarum*-induced IFN- β production during the late stages of infection

To test whether increased STAT1 expression and activation contributed to *C. muridarum*-induced IFN- β responses in OE cells, OEB6 WT and OE STAT1 (–/–) cells that were derived from WT and STAT1-deficient mice of the C57BL/6 genetic background, respectively, were infected with *C. muridarum* and supernatant IFN- β levels were measured by ELISA every 4 h, up until 24 h postinfection (Fig. 2). As shown, IFN- β was secreted in increasing concentrations into the supernatants of the OEB6 WT cells, whereas IFN- β production was significantly diminished in the *Chlamydia* infected OE STAT1 (–/–) cells after the 12 h PI time point. We observed similar results using bone marrow-derived macrophages (BMDMs) from WT and STAT1-deficient mice, whereas the BMDMs deficient in STAT1 expression were also diminished in their ability to produce similar levels of IFN- β in response to progressive *C. muridarum* infection (data not shown).

TABLE 1. PRIMERS FOR RT-PCR AND RT-qPCR

	Sense primer	Antisense primer	Product size (bp)
IFN- β	5'-AAGAGTTACACTGCCTTTGCCATC-3'	5'-CACTGTCTGCTGGTGGAGTTCATC-3'	110
IFN- $\alpha 2$	5'-AGCAGATCCAGAAGGCTCAA-3'	5'-CATTCCAAGCAGCAGATGAA-3'	99
IFN- $\alpha 4$	5'-TTCTGCAATGACCTCCATCA-3'	5'-TATGTCCTCACAGCCAGCAG-3'	101
STAT1	5'-CGGAGTCGGAGGCCCTAAT-3'	5'-ACAGCAGGTGCTTCTTAATGAG-3'	140
STAT2	5'-TTTGGCTACCTGGATTGAAGAC-3'	5'-GGCTGAATTTTCGCAAGTTATGC-3'	170
IRF7	5'-CAATTCAGGGGATCCAGTTG-3'	5'-AGCATTGCTGAGGCTCACTT-3'	112
β -Actin	5'-GGCTGTATTCCCCTCCATCG-3'	5'-CCAGTTGGTAACAATGCCATGT-3'	154

FIG. 1. *Chlamydia muridarum*-induced STAT1 expression and activation in oviduct epithelial (OE) cells. OE129 WT cells and OEB6 WT cells were mock-infected, treated with either recombinant interferon γ (IFN- γ) at 10 ng/mL for 6 h, or infected with 10 MOI of *C. muridarum* (MoPn) for 24 h. (A) Total cell RNA was extracted, reverse transcribed, and amplified with STAT1 primers and β -actin (loading control). (B) Total cell lysates were subjected to western blot analysis to evaluate total STAT1 protein expression and phosphorylation. β -Actin served as a loading control. (C) Densitometry of total STAT1 protein expression and phosphorylation in OE129 WT and OEB6 WT cells. The results shown are representative of 3 independent experiments.

FIG. 2. *C. muridarum*-induced IFN- β production is diminished in STAT1-deficient OE cells. OEB6 WT cells and OESTAT1 (-/-) cells were mock-infected and/or infected with 10 MOI of *C. muridarum* for a total of 24 h. ELISA was used to measure infection-induced IFN- β secreted into the supernatants of the *C. muridarum*-infected OE cells every 4 h. The data represent mean $\pm$ SD and are representative of 3 different experiments conducted in triplicate. Significance was determined using Student's *t*-test. **P* value < 0.05; ***P* value < 0.005 for *C. muridarum* infected OEB6 WT cells versus OESTAT1 (-/-) cells.

STAT1 is critical for regulating *in vitro* *C. muridarum* replication/growth in OE cells

It has been shown that *C. trachomatis* can cause an upregulation and activation of the JAK/STAT signaling pathway, which resulted in restricting chlamydial replication in HeLa 229 cells (Lad and others 2005). To ascertain whether *C. muridarum*'s ability to induce STAT1 protein expression and phosphorylation in OE cells has any effect on chlamydial pathogenesis, we investigated the *in vitro* effects of STAT1 on *C. muridarum* growth and replication in OE cells. OEB6 WT and OE STAT1 (-/-) cells were either mock-infected or infected with 10 IFU/cell *C. muridarum* for 24 h, and *Chlamydia* inclusion bodies were fluorescently stained using antibody specific for chlamydial LPS. As shown, *Chlamydia* inclusions were detected in many of the *C. muridarum*-infected OEB6 WT cells as fluorescent small round inclusions within the cell when compared to mock-infected OEB6 WT controls (Fig. 3A). However, the STAT1-deficient OE cells displayed aberrantly shaped and comparatively larger *Chlamydia* inclusions that appeared to span the entire area of the cell cytoplasm, and the inclusions were detected in most of the OE STAT1 (-/-) cells. The larger inclusions imply that there are increased numbers of chlamydial progeny in these cells, suggesting that STAT1 plays a critical role in restricting *C. muridarum* replication in OE cells.

FIG. 3. *C. muridarum*-infected STAT1-deficient OE cells display aberrantly shaped *Chlamydia* inclusion-forming units (IFU); implicating uncontrolled *C. muridarum* replication. (A) OEB6 WT and OE STAT1 (-/-) cells were mock-infected, or infected with 10 MOI of *C. muridarum* (MoPn) for 24 h. All cells were stained with a genus-specific anti-LPS antibody, and counterstained with a FITC-labeled anti-mouse IgG antibody for detection of *Chlamydia* IFUs via fluorescent microscopy. (B) *Chlamydia* replication in OE cells derived from wild-type (WT) and STAT1-deficient mice. OE cells were infected with 5 IFU of *C. muridarum*/cell. At 30 h post-infection, lysates were collected, sonicated, and titered on McCoy cell monolayers to determine viable *Chlamydia* in each OE cell type. The data presented are representative from 3 different experiments. Significance was determined using the Student's *t*-test (** $P < 0.005$). Original magnification was at 100 $\times$. Chlamydia IB, Chlamydia inclusion body.

To examine whether *C. muridarum* growth is actually affected by the presence of STAT1 in OE cells, we next assessed *C. muridarum* replication in WT and STAT1-deficient OE cells. As shown in Fig. 3B, chlamydial progeny was significantly higher in the OE STAT1 (-/-) cells compared with the WT control OE cells, suggesting that STAT1-deficiency negatively affects the control of *Chlamydia* replication in the OE cells. Collectively, our findings in OE cells support the observations of Lad and others (2005) who demonstrate a role for STAT1 in restricting *Chlamydia* growth in HeLa 229 cells.

STAT1 protein activation/expression and gene expression is disrupted in TLR3-deficient OE cells infected with *C. muridarum*

We previously reported that *C. muridarum*-induced IFN- β production in OE cells was mostly dependent on TLR3

signaling (Derbigny and others 2010). To assess the role of STAT1 in *Chlamydia*-induced IFN- β production and how it relates to TLR3 deficiency in OE cells, OE129 WT and OE TLR3 (-/-) cells [derived from WT and TLR3-deficient mice of the 129S1 genetic background (Derbigny and others 2010)] were either mock-infected, IFN- γ -treated, or infected with 10 IFU/cell *C. muridarum* before analyses for STAT1 gene expression and protein activation. As shown in Fig. 4A, *C. muridarum* infection significantly increased STAT1 mRNA levels in OE129 WT cells when compared to mock-infected controls. However, the STAT1 mRNA induction during *C. muridarum* infection was substantially diminished in OE TLR3 (-/-) cells when compared with the OE129 WT cells. We performed western blot analysis to validate the RT-PCR data (Fig. 4B). As shown, the OE129 WT cells exhibited significant increases in both total STAT1 expression and activation (as compared to mock-infected controls) upon *Chlamydia* infection, and the total STAT1 levels were comparable to IFN- γ -treated controls (Fig. 4C). However, the notably decreased induction of STAT1 mRNA in *C. muridarum*-infected OE TLR3 (-/-) cells, correlated to severely decreased levels of total STAT1 protein synthesis and activation upon *C. muridarum* infection when compared to OE129 WT cells and the IFN- γ -treated controls (Fig. 4B, C).

STAT1 is essential for upregulating IFN- β transcription during late-stage *C. muridarum* infection

To address the role of STAT1 in sustaining and amplifying *Chlamydia*-induced type-1 IFN production in OE cells and to delineate its role as either an early- or late-stage factor in the synthesis of type-1 IFNs during infection, we infected WT, STAT1-deficient, and TLR3-deficient OE cells with *C. muridarum*, and isolated total cell mRNA at either 6 h (early-stage) or 16 h (late-stage) time points. As shown in Fig. 5A, *C. muridarum* induced IFN- β gene expression in both WT OE cells lines and the TLR3-deficient OE cells at 6 h PI, and this induction was significantly increased in all cell lines at the 16 h time point. The 8- and 10-fold higher induction of the IFN- β gene at late infection in the WT OE cells were substantially greater than the 2-fold induction late in the OE TLR3 (-/-) cells, which corroborates our previous studies describing the importance of TLR3 in the *Chlamydia*-induced synthesis of IFN- β in OE cells (Derbigny and others 2010). Although we noted a higher relative transcription level of the IFN- β gene in the OE 129WT cells when compared to the OEB6 WT cells, all of the other genes we analyzed in these studies were basically equivalent in their levels of gene transcription; which suggest a similar immune response to *Chlamydia* infection in the oviducts of these 2 WT mouse strains. Interestingly, IFN- β transcription was only modestly induced (~ 2 -fold) at the 6 h time point in the OE STAT1 (-/-) cells; however, there was no further induction of the IFN- β gene at the 16 h time point in the STAT1-deficient OE cells. These findings suggest that STAT1 has some limited role in the synthesis of IFN- β at early times; however, its function in upregulating IFN- β synthesis at late times during infection appears to be indispensable.

FIG. 4. STAT1 expression and activation is diminished in TLR3-deficient OE cells during *C. muridarum* infection. OE129 WT cells and OE TLR3 ($-/-$) cells were mock-infected, treated with IFN- γ at 10 ng/mL for 6 h, or infected with 10 MOI of *C. muridarum* (MoPn) for 24 h. (A) Total cell RNA was extracted, reverse transcribed, and amplified with STAT1 primers and β -actin (loading control). (B) Total cell lysates were subjected to western blot analysis to evaluate total STAT1 protein expression and phosphorylation. β -Actin served as a loading control. (C) Densitometry of total STAT1 protein expression and phosphorylation in OE129 WT and TLR3 ($-/-$) OE cells. The results shown are representative of 3 independent experiments.

Figure 5B–D show results of the *Chlamydia*-induced gene induction of signaling mediators found within the type-1 IFN signaling pathway. As shown, both *Stat2* and *IRF7* genes were induced at similar levels in the WT and TLR3-deficient OE cells; suggesting that the type-1 IFN signaling pathway is intact and induced at similar levels, despite the substantial difference in IFN- β synthesis during *Chlamydia* infection. OE cells deficient in STAT1 showed essentially no upregulation in either *Stat2* or *IRF7* implicating a critical role for STAT1 in the inductions of genes found in the type-1 IFN signaling pathway. Interestingly, *C. muridarum* mediated *Stat1* gene induction at 16 h PI (Fig. 5B) was lower in the TLR3-deficient cells than the 2 WT OE cell lines, despite almost equal levels in the gene induction of *Stat2* and *IRF7*. The almost 2-fold more induction of the *Stat1* transcription in the WT versus TLR3-deficient OE cells, proposes a role for STAT1 in the *Chlamydia*-induced synthesis of IFN- β that appears to be partly reliant on the TLR3-dependent IFN- β produced during the course of infection. However, because there were equivalent levels of induction of other genes found in the type-1 IFN signaling pathway (*IRF7* and *Stat2*), our data implicate a secondary function for STAT1 in the *Chlamydia*-induced IFN- β production that is independent of its role in the type-1 IFN signaling pathway, and that this secondary function of STAT1 is likely attenuated in TLR3-deficient OE cells.

C. muridarum-induced IFN- α production is highly dependent on STAT1 early during infection, but is differentially dependent on STAT1 at late times during infection

Our data propose a critical role for STAT1 in the optimal synthesis of IFN- β during *Chlamydia* infection of OE cells, and that this role occurs through a pathway that is hypothesized to be independent of the type-1 IFN signaling pathway. To ascertain whether STAT1 has a role during the *Chlamydia*-induced synthesis of other type-1 IFNs in OE cells, we measured the transcription levels of candidate IFN α genes that were induced at 6 and 16 h PI. Figure 6 shows RT-qPCR results for analyses of IFN α -2 and IFN α -4 gene transcription in WT and STAT1-deficient OE cells and as indicated, *C. muridarum* induced expression of both genes throughout the course of infection. Interestingly, IFN α -2 was induced at higher levels early during infection, whereas IFN α -4 appeared to be expressed at higher levels late during *Chlamydia* infection of WT OE cells. Synthesis of both genes were highly dependent on STAT1 early during infection; however, there was an even higher induction of IFN α -2 gene transcription late during *Chlamydia* infection in the STAT1-deficient OE cells. These findings suggest that *C. muridarum*-induced IFN α -2 gene expression can also occur through STAT1-independent pathways late during

FIG. 5. *C. muridarum* infection induces expression of IFN- β and components of the type-1 IFN signaling pathway. OE129 WT, OE B6WT, OE TLR3 (-/-), and OE STAT1 (-/-) cells were either mock-infected, or infected with 10 MOI of *C. muridarum* (MoPn) for either 6 h (white bars) or 16 h (black bars). Quantitative real-time polymerase chain reaction (PCR) was performed to measure (A) IFN- β , (B) STAT1, (C) STAT2, and (D) IRF7 gene expression, using 1 μ g of total-cell mRNA isolated from each cell type for each condition with the respective primers (Table 1). Control reactions were set up with β -actin primers to ensure equal loading of RNA. The results are presented as mean fold change $\pm$ SD; and are representative data from 1 of 3 individual experiments conducted in triplicate. *ND=not done.

FIG. 6. *C. muridarum* induced IFN- α gene transcription in WT and STAT1-deficient OE cells. OE B6WT and OE STAT1 (-/-) cells were either mock-infected, or infected with 10 MOI of *C. muridarum* (MoPn) for either 6 h (white bars) or 16 h (black bars). Quantitative real-time PCR was performed to measure (A) IFN- α 2 and (B) IFN- α 4 gene expression, using 1 μ g of total-cell mRNA isolated from each cell type for each condition with the respective primers (Table 1). Control reactions were set up with β -actin primers to ensure equal loading of RNA. The results are presented as mean fold change $\pm$ SD; and are representative data from 1 of 3 individual experiments conducted in triplicate.

infection, and imply that STAT1 can differentially regulate expression of IFN α -2 late during infection.

C. muridarum-induced IFN- α production is dependent on the type-1 signaling pathway in OE cells

Our data show that IFN- α is synthesized during *Chlamydia* infection of OE cells, and that STAT1 has some function in regulating its synthesis. One potential mechanism in which STAT1 can have a role in type-1 IFN synthesis is through JAK-STAT signaling via the type-1 IFN signaling pathway that is triggered when interferon- α/β receptor1 (IFNAR1) binds to the appropriate ligand (Decker and others 2002; Dupuis and others 2003). To examine whether the type-1 IFN pathway is involved in the *Chlamydia*-induced IFN- α production in OE cells, we measured the transcription of candidate IFN- α genes induced by *C. muridarum* at 6 and 16 h PI (see the Methods section), in the presence or absence of neutralizing antibody specific for IFNAR1 (Fig. 7).

As shown in Fig. 7A, *C. muridarum* infection induced expression of the IFN α -2 gene in WT and TLR3-deficient OE cells, at early and late times during infection, although the OE cells made substantially more IFN α -2 early in the infection (6 h) in both cell types. In contrast to what was seen in the induction of the IFN- β during *C. muridarum* infection (Fig. 5), the induction of IFN α -2 appeared to be attenuated by the presence of TLR3 in WT OE cells (note

the 40-fold higher induction of the IFN α -2 gene in the TLR3-deficient OE cells). These data suggest a possible negative regulation of IFN α -2 gene expression by TLR3 signaling during *Chlamydia* infection. Blocking the type-1 IFN pathway with IFNAR1 neutralizing antibody led to significant reductions in the *C. muridarum*-induced synthesis of IFN α -2 in WT and TLR3-deficient OE cells at early and late infection, implicating an important role for IFNAR1-mediated signaling through the type-1 IFN pathway in the *C. muridarum*-induced synthesis of IFN α -2.

We next examined the role of the type-1 IFN signaling pathway on the expression of IFN α -4. As shown in Fig. 7B, *C. muridarum* infection induced IFN α -4 gene expression at 6 and 16 h PI in both WT and TLR3-deficient OE cells. However, in contrast to what was observed with IFN α -2 gene expression, there were higher levels of IFN α -4 gene transcription late (16 h) in infection. Interestingly, blocking the type-1 IFN pathway with neutralizing antibody specific for IFNAR1 led to significant reductions in WT OE cells at the 16 h time point, but not at the 6 h time point. In contrast to what was observed in the OE129 WT cells, blocking the type-1 IFN pathway with neutralizing antibody specific for IFNAR1 led to significant reductions in IFN α -4 gene induction at both time points in TLR3-deficient OE cells. Also, stimulation of the TLR3 signaling pathway during *Chlamydia* infection appears to attenuate the expression of IFN α -4 in the OE cells, which is demonstrated by the >15-fold more induction of IFN α -4 in the OE TLR3 (-/-) cells versus the OE129 WT cells.

FIG. 7. Temporal and differential upregulation of *C. muridarum*-induced IFN- α gene subtypes requires IFNAR1 and is attenuated by TLR3. OE129 WT and OE TLR3 (-/-) cells were either mock-infected, or infected with 10 MOI of *C. muridarum* (MoPn) for either 6 h (white bars) or 16 h (black bars) with or without IFNAR1 neutralizing antibody (denoted as α -IFNAR; see the Methods section). Quantitative real-time PCR to measure (A) IFN α -2 mRNA and (B) IFN α -4 mRNA was performed using 1 μ g of total-cell RNA isolated from each cell type for each condition with the respective primers (Table 1). Control reactions were set up with β -actin primers to ensure equal loading of RNA. The results are presented as mean fold change $\pm$ SD; and are representative data from 1 of 3 individual experiments conducted in triplicate. **P* value < 0.05; ***P* value < 0.005; NS, not statistically significant.

FIG. 8. The STAT1-dependent autocrine/paracrine induction of IFN- α occurs via IFNAR1 signaling pathways. OEB6 WT and OE STAT1 (-/-) cells were either mock-treated, or treated with exogenous IFN- β in the presence or absence of either IFNAR1 neutralizing antibody (denoted as α -IFNAR) or the isotype control (denoted as α -IgG), quantitative real-time PCR to measure: (A) IFN α -2 mRNA and (B) IFN α -4 mRNA was performed using 1 μ g of total-cell RNA isolated from each cell type for each condition with the respective primers (Table 1). Control reactions were set up with β -actin primers to ensure equal loading of RNA. The results are presented as mean fold change $\pm$ SD; and are representative data from 1 of 3 individual experiments conducted in triplicate. * P value < 0.05; ** P value < 0.005; NS, not statistically significant.

The autocrine/paracrine induction of IFN- α is only partly dependent on STAT1 and IFNAR1-mediated type-1 IFN signaling pathways in OE cells

Our results suggest a hypothesis that IFN- β is induced early during *C. muridarum* infection of OE cells through mostly TLR3-dependent mechanisms, and that the secreted IFN- β enhances the expression of other type-1 IFNs via autocrine/paracrine mechanisms that signal through IFNAR1. Our data implicate the involvement of STAT1 in the autocrine/paracrine synthesis of type-1 IFN, likely as a part of JAK-STAT signaling pathways. We sought to ascertain the impact of IFNAR1-mediated signaling pathways in the autocrine/paracrine transcription of IFN- α by exogenous recombinant IFN- β , and to determine whether this pathway is dependent on functional STAT1 protein in OE cells. We measured the transcription rates of IFN α -2 and IFN α -4 in WT and STAT1-deficient OE cells that were pretreated with 50 U/mL recombinant IFN- β at times representing early (6 h) and late (16 h) *Chlamydia* infection (Fig. 8). To determine whether IFNAR1 has a role in the autocrine/paracrine transcription of IFN- α in OE cells, the transcription rates were measured while blocking the type-1 IFN pathway with IFNAR1-specific neutralizing antibody. As shown in Fig. 8A, we were able to completely block IFN- β induced transcription of IFN α -2 at the 6 h time point with IFNAR1-specific antibody; however, the autocrine-paracrine-induced transcription of IFN α -2 at the 16 h time point was not blocked by the antibody and appeared to occur via STAT1-independent mechanisms. These findings corroborate the *Chlamydia* infection data that also show a STAT1-independent transcription of IFN α -2 late during *C. muridarum* infection of OE cells (Fig. 6A). In contrast, synthesis of IFN α -4 appeared to be completely

dependent on having a functional STAT1 protein in OE cells, and appeared to signal through IFNAR1-mediated signaling pathways at both time points during autocrine-paracrine induction by exogenous recombinant IFN- β (Fig. 8B).

We next examined the impact (if any) that TLR3 signaling pathways have the autocrine-paracrine induction of IFN- α transcription by exogenous recombinant IFN- β , and we sought to determine whether IFNAR1-mediated signaling pathways were affected by TLR3 function (Fig. 9). As indicated, WT OE cells treated with recombinant IFN- β showed substantial inductions in the gene expression of IFN α -2, but only modest induction of IFN α -4 at the 6 h time point. Blocking the type-1 IFN pathway with antibody specific for IFNAR1 led to reductions in the transcription of both IFN α -2 and IFN α -4 genes in the OE129 WT cells at the 6 h time point; however, there was no impact on either IFN α -2 or IFN α -4 transcription at the 16 h time point using blocking antibody to IFNAR1. Transcription of IFN α -2 and IFN α -4 was induced at both time points in the TLR3-deficient OE cells, and transcription was significantly reduced by IFNAR1-specific neutralizing antibody at both time points. Interestingly, TLR3-deficient OE cells showed a much more modest increase in transcription of IFN α -2 and IFN α -4 in response to recombinant IFN- β when compared to the WT OE cells ($\sim$ 2-fold). This relatively small increase in IFN α transcription in the TLR3-deficient OE cells contrasts the *C. muridarum* infection results, which demonstrated a substantially higher fold-induction of IFN α -2 and IFN α -4 in the OE TLR3 (-/-) cells (40- and 15-fold, respectively; Fig. 7). The huge difference in IFN- α gene transcription between *C. muridarum* infection of WT versus TLR3-deficient OE cells reveal that *Chlamydia*-induced IFN- α transcription is dramatically attenuated by TLR3

FIG. 9. Autocrine/paracrine induction of IFN- α does not require TLR3 signaling. OE129 WT and OE TLR3 (-/-) cells were either mock-treated, or treated with exogenous IFN- β in the presence or absence of either IFNAR1 neutralizing antibody (denoted as α -IFNAR) or the isotype control (denoted as α -IgG), quantitative real-time PCR to measure (A) IFN α -2 mRNA and (B) IFN α -4 mRNA was performed using 1 μ g of total-cell RNA isolated from each cell type for each condition with the respective primers (Table 1). Control reactions were set up with β -actin primers to ensure equal loading of RNA. The results are presented as mean fold change $\pm$ SD; and are representative data from 1 of 3 individual experiments conducted in triplicate. * P -value < 0.05 ; ** P -value < 0.005 ; NS, not statistically significant.

signaling triggered by chlamydial PAMPs during infection. In contrast, there was a much more modest increase in autocrine/paracrine induction of IFN- α by IFN- β in TLR3-deficient versus WT OE cells. Our findings demonstrate that IFN- α transcription triggered by autocrine-paracrine induction by exogenous recombinant IFN- β is not substantially affected by the presence of TLR3, suggesting that the TLR3 signaling pathway likely has no function during the autocrine/paracrine response to IFN- β .

STAT1 is required for the optimal induction of IFN- β gene transcription in OE cells during Chlamydia infection, but it functions through pathways that do not signal through IFNAR1

We explored the function of IFNAR1 during the transcription of IFN- β throughout *Chlamydia* infection of OE cells, and we wanted to ascertain whether there was a role for IFNAR1 in the autocrine/paracrine regulation of IFN- β gene in response to exogenous recombinant IFN- β (Fig. 10). Contrary to what was observed in the *C. muridarum*-induced synthesis of IFN- α in OE cells, blocking the type-1 IFN signaling pathway with IFNAR1-specific antibody had no effect on the induction of the IFN- β gene transcription by either *Chlamydia* infection or by autocrine-paracrine induction using exogenous recombinant IFN- β (Fig. 8A, C). As shown, the WT OE cells had significant upregulation in the expression of the IFN- β gene in response to *Chlamydia* infection and treatment with exogenous recombinant IFN- β . However, there was no significant reduction in the level of IFN- β gene transcription when IFNAR1 was blocked with specific antibody.

These data differ from the IFN- α gene expression experiments, which demonstrated at least some dependence on

IFNAR1 during *C. muridarum* infection and in the autocrine-paracrine induced transcription of both IFN α -2 and IFN α -4 (Figs. 8 and 9). We addressed the unlikely possibility of the antibody not being functional by measuring both IFN- β and IFN- α transcription simultaneously in these experiments (Figs. 5–10), and the IFN- α results suggest that IFNAR1 function is at least partially blocked by the addition of the neutralizing antibody. Similar to what was observed in the transcription of IFN- α , the data from Figs. 5A and 10B and D show a high reliance on STAT1 for the induction IFN- β transcription; particularly at 16 h time points.

Collectively, our data show that STAT1 has an important role in the optimal synthesis of type-1 IFN during the course of *Chlamydia* infection. Our findings suggest that IFN- α and IFN- β are both inversely affected by TLR3 signaling during *C. muridarum* infection of OE cells, but that TLR3 likely has no role in the autocrine-paracrine synthesis of type-1 IFN during infection. Finally, our data demonstrate that type-1 IFNs induced by *Chlamydia* infection of OE cells are only differentially dependent on IFNAR1-mediated signaling pathways.

Discussion

The epithelial cells' innate immune responses play an important role in the onset of pathogenesis during *Chlamydia* infection; however, these immune responses are critical for effective clearance of the pathogen (Rasmussen and others 1997; Stephens 2003). Here, we show that STAT1, a key component of type-1 IFN signaling pathway, is critical for optimal *Chlamydia*-induced IFN- β production in OE cells. We also show that STAT1 plays a major role in the *Chlamydia*-induced synthesis of other type-1 IFNs in OE cells throughout the course of infection. Although we show that the STAT1-dependent IFN- α synthesis occurs mostly

FIG. 10. IFNAR1 is not involved in the synthesis of IFN- β during *Chlamydia* infection of OE cells. **(A)** OE6 WT and **(B)** OE STAT1 (-/-) cells were mock-infected or infected with 10 MOI of *C. muridarum* (MoPn) for either 6 h (white bars) or 16 h (black bars) with or without IFNAR1 neutralizing antibody (denoted as α -IFNAR). To examine the role of IFNAR in autocrine/paracrine induction of IFN- β , **(C)** OE6 WT and **(D)** OE STAT1 (-/-) cells were either mock-treated, or treated with exogenous IFN- β in the presence or absence of either IFNAR1 neutralizing antibody (denoted as α -IFNAR), or the isotype control (denoted as α -IgG). Quantitative real-time PCR to measure IFN- β mRNA levels was performed on 1 μ g of total-cell RNA for each condition with IFN- β primers (Table 1). Control reactions were set up with β -actin primers to ensure equal loading of RNA. The results are presented as mean fold change $\pm$ SD; and are representative data from 1 of 3 individual experiments conducted in duplicate. NS, not statistically significant.

via IFNAR signaling in *Chlamydia*-infected OE cells, our data suggest that STAT1 functions in the synthesis of IFN- β through pathways that are independent of IFNAR signaling. Furthermore, we report on a unique expression pattern of type-1 IFNs during *C. muridarum* infection by demonstrating a differential regulation and dependence on TLR3 that differs between IFN- β and other type-1 IFNs.

We showed that STAT1 gene expression, protein production, and protein activation were significantly increased during *Chlamydia* infection in WT OE cells derived from 2 different mouse strains. Interestingly, our results showed that STAT1 was induced in the 2 WT OE cell lines, and thereby suggesting a similar cellular mechanism involving STAT1 gene and protein function is triggered by *Chlamydia* infection in this specific cell type. We routinely saw that the same set of inflammatory genes were induced during *Chlamydia* infection of OE cells derived from the 2 different mouse strains used in these experiments, and thereby corroborating our hypothesis that *Chlamydia* infection triggers other cell signaling events through similar pathways in the same cell type.

However, the level of gene induction varied between the 2 different mouse strains, which likely contributes to the variances in disease progression and associated pathologies observed in different mouse strains when infected with the same chlamydial pathogen (Pal and others 2003; Lyons and others 2005; Chen and others 2014). Despite the measurable differences we saw in gene transcription and inflammatory cytokine syntheses in the OE cells derived from the 2 mouse strains, the overall similarities in the kinetics of induction allows us to extrapolate that the findings in the OE cells derived from one mouse strain will likely be representative of results of OE cells derived from mice from other genetic backgrounds.

We provide evidence suggesting that STAT1 is critical for regulating IFN- β secretion and restricting *C. muridarum* replication in OE cells (Figs. 2 and 3, respectively). STAT1 is well established as a mediator involved in type-1 IFN signaling and type-1 IFN-dependent antiviral and antimicrobial responses (Decker and others 2002; Dupuis and others 2003). Additionally, STAT1 has been implicated as a critical regulator of type-1 and type-2 IFN-dependent control of *Chlamydia* infections in nonhematopoietic cells (Lad and others 2005; Rothfuchs and others 2006). Lad and others demonstrated that HeLa 229 epithelial cells infected with *C. trachomatis* showed an increased STAT1 activation, with a resultant reduction in *Chlamydia* replication compared with STAT1-null and/or STAT1 knockdown cells. In that study, they showed that inactivating the effects of IFN- β with neutralizing antibody resulted in decreased STAT1 activation; thereby suggesting that STAT1 activity was IFN- β -dependent in cervical epithelial cells, and proposing that STAT1 has a role in the IFN- β autocrine/paracrine regulatory pathway.

By seeking to determine whether STAT1 contributed to the residual amount of *Chlamydia*-induced IFN- β in OE cells lacking TLR3, this study's investigation into the role of STAT1 sought to further define the role of STAT1 in the pathogenesis of *Chlamydia* infection. The results shown in Fig. 5A detail the significant reduction in IFN- β gene expression during *C. muridarum* infection of OE cells lacking TLR3 compared to WT OE cells; however, the lack of STAT1 appeared to have a more dramatic effect in reducing IFN- β synthesis, particularly late during infection. These findings were corroborated in STAT1 knockdown experiments in which we observed reductions in *C. muridarum*-induced IFN- β synthesis when WT OE cells were pretreated STAT1-specific

si-RNA before infection (data not shown). Because STAT1 is a major component found in the type-1 IFN signaling pathway, the data implicate an important role for the type-1 IFN signaling pathway in the synthesis of IFN- β during *Chlamydia* infection of OE cells, and proposes a critical role for IFNAR-dependent type-1 IFN signaling pathways in the synthesis of type-1 IFNs during *Chlamydia* infection of OE cells.

We showed that IFNAR1 had a major role in the synthesis of IFN- α throughout the course of *Chlamydia* infection by demonstrating significant reductions in *Chlamydia*-induced IFN- α transcription when IFNAR1-mediated signaling was blocked with neutralizing antibody. However, we showed that IFNAR1-dependent type-1 IFN signaling pathways had virtually no effect on the synthesis of IFN- β during *C. muridarum* infection of OE cells in parallel experiments where IFNAR1 was blocked by neutralization antibody (Fig. 10). Also, the autocrine/paracrine gene induction of IFN- β in response to exogenous IFN- β appeared to be completely unaffected by neutralization antibody to the IFNAR1, whereas both IFN α -2 and IFN α -4 showed significant but differential reductions in their transcription induction by exogenous IFN- β when the IFNAR1 was blocked with neutralization antibody. Although all 3 type-1 IFNs are highly dependent on STAT1 for their optimal syntheses in response to *C. muridarum* infection, our data suggest that the *Chlamydia*-induced synthesis of IFN- β in OE cells is regulated by STAT1-dependent pathways that are distinct from the type-1 IFN signaling pathways that require IFNAR1.

Our data propose that STAT1 is critical for the optimal synthesis of IFN- β in OE cells during *Chlamydia* infection, and that the STAT1 activation occurs via mechanisms that do not involve the IFNAR-mediated signaling pathways that are required for synthesis of other type-1 IFNs. It is possible that *Chlamydia* instead activates STAT1 through direct stimulation of TLRs, to synthesize immune factors, which are known to activate JAK/STAT signaling pathways that are independent of the IFNAR. One such immune factor that can activate JAK/STAT signaling via an alternative pathway is IL-6.

We previously showed that *C. muridarum* induces IL-6 in a TLR2-dependent manner in OE cells; and though it can be detected as constitutively expressed in the supernatants of mock-treated cells, IL-6 is significantly induced by *Chlamydia* infection at significantly higher levels that can be detected in the supernatants of infected cells as early as 3 h PI (Johnson 2004; Derbigny and others 2005). Others have described activation of JAK/STAT signaling pathways through IL-6 binding to the IL-6 receptor in human fibrosarcoma cells, resulting in the upregulation of IRF1 gene transcription and potentially inducing type-1 IFN production (Guschin and others 1995). It is possible that upon initially encountering *Chlamydia*, the induction of TLR2-dependent IL-6 leads to an activation of STAT1-dependent IRF1 induction, which then serves as a primer or co-activator for more robust TLR3-dependent IFN- β synthesis in OE cells. Our results show that *C. muridarum* can upregulate the expression of IFN- β 2-fold in absence of STAT1 in the OE cells; however, this minimal upregulation is much less than what occurs in either the WT or TLR3-deficient OE cells (Fig. 5A). The relationship between STAT1 deficiency and IL-6 receptor-dependent IRF1 gene transcription has not yet been investigated in OE cells, and its impact on the synthesis of *Chlamydia*-induced IFN- β requires further investigation.

The role of IFN- α has been extensively studied and well documented in viral infections (Jonas and others 1998; Takeda and others 2003; Bourne and others 2007; Szabo and Dolganiuc 2008; Barbe and others 2010; Trinchieri 2010); however, the regulatory mechanisms governing IFN- α production in *Chlamydia* infections and other similar intracellular bacterial pathogens are poorly understood. Our data revealed that there were notable increases in IFN α -2 and IFN α -4 gene expression in WT OE cells upon *C. muridarum* infection, and that both subtypes were induced at similar levels in a STAT1-dependent manner. Interestingly, IFN α -2 was mostly induced at the early stage of infection, while IFN α -4 was induced at a significantly higher level during the late-stage of infection. Both IFN- α subtypes' gene transcription levels were substantially reduced upon inhibition of type-1 IFN signaling by IFNAR1-specific neutralizing antibody, implicating the importance of this pathway in their *Chlamydia*-induced syntheses. Our results reveal a suspected differential induction of IFN- α subtypes in OE cells during *C. muridarum* infection; however, our results differs from the investigations of others, which typically identify IFN α -4 as the "early subtype" and refer to IFN α -2 as one of the "late subtypes" (Hoss-Homfeld and others 1989; Marie and others 1998; Le and others 2008). Nevertheless, other studies demonstrate that the temporal induction of these subtypes can vary dependent on cell types and modes of induction (viral versus bacterial) (Raymond and others 1992; Hillyer and others 2012).

We showed that both IFN α -2 and IFN α -4 were induced in response to exogenous IFN- β , supporting our hypothesis that *Chlamydia*-induced IFN- β regulates the synthesis of other type-1 IFNs during infection. These data support the investigations of others showing that IFN- β -deficient macrophages produce significantly reduced levels of IFN- α in response to *C. pneumoniae* infection (Trumstedt and others 2007). Interestingly, we showed that OE cells lacking TLR3 induced the IFN α -2 and IFN α -4 gene transcription at a considerably higher level (40- and 25-fold, respectively; Fig. 7) when compared to WT. These results suggest that TLR3 plays a role in regulating the expression of IFN α , particularly by attenuating its synthesis during infection.

Recent studies have identified a plausible mechanism for attenuation of several chemokines and cytokines (including type-1 IFN), that is dependent on TLR signaling in murine macrophages. One such mechanism involves a TLR-dependent induction of the scaffold/adaptor protein, p62 (Kim and Ozato 2009). Although it is unclear exactly whether TLR3 stimulation would lead to p62 activation during *Chlamydia* infection of OE cells, the ability of TLR3 to attenuate expression of IFN- α via ubiquitin modification by way of p62 activity would suggest that TLR3 is involved in a process to prevent excessive inflammatory responses following pathogen/stress signaling. If the synthesis of certain type-1 IFNs is indeed detrimental to the host during *Chlamydia* infection (Nagarajan and others 2008), then TLR3's function in this manner would represent an important role for TLR3 in *Chlamydia* pathogenesis because of its potential to limit genital tract damage. This would support our ongoing hypothesis that TLR3 has a critical role in controlling the outcome of *Chlamydia* infection in OE cells because of its ability to modulate the expression of numerous innate immune factors (Derbigny and others 2012). In those previous studies, we described TLR3's ability to

FIG. 11. Proposed model of type-I IFN induction during *Chlamydia* infection of OE cells. During early infection of murine OE cells: **(A)** *Chlamydia* replication triggers early IFN- β synthesis by mostly TLR3-dependent mechanism (I), while having some minor synthesis through putative TLR3-independent mechanisms (II) in WT OE cells. Synthesis of other TLR3-dependent gene products such as our hypothesized “interferon regulator” (p62?) protein (III) is synthesized through unknown signaling intermediates. **(B)** *Chlamydia* replication triggers significantly reduced amounts of IFN- β synthesis through TLR3-independent mechanisms (II) in TLR3-deficient OE cells, but will not express the hypothesized TLR3-dependent “interferon regulator” protein. **(C)** During mid-infection of murine OE cells, the TLR3-dependent and TLR3-independent IFN- β binds to the IFNAR complex (IV) to induce synthesis of IFN α -2, IFN α -4, and possibly IFN- β via STAT1 and IRF7-dependent signaling mechanisms. The hypothesized “interferon regulator” protein modulates the activity of IFNAR to regulate IFN- α synthesis in WT cells, whereas its absence in OE-TLR3^{-/-} cells results in significantly higher expression levels of IFN- α in TLR3-deficient cells (Fig. 7). The expression of the proposed IFN- β receptor (V) is induced during early-mid infection through unknown mechanisms by the presence of chlamydial PAMPs during the bacterial replication cycle. **(D)** During late-infection of murine OE cells, the IFN- α and IFN- β induced early- and mid-infection induces autocrine/paracrine expression of type-1 IFN via IFNAR (VI); however, the majority of the autocrine/paracrine induction of IFN- β during *Chlamydia* infection is induced via the proposed IFN- β receptor (VII), which functions through STAT1 and IRF7-through either known or unknown signaling pathways.

modulate the innate-immune response during *C. muridarum* infection in OE cells, using mechanisms that are dependent and are independent of IFN- β synthesis. Future studies will investigate whether activation of the p62 scaffold/adaptor protein is diminished or enhanced in absence of TLR3 signaling, and whether its function is regulated by IFN- β -dependent mechanisms.

Finally, our data propose a unique mechanism for the regulation of IFN- β during *Chlamydia* infection of murine OE cells, which does not quite fit the known paradigm that type-I IFNs are induced via signaling through IFNAR. In this preliminary investigation into the relationship between TLR3-induced IFN- β synthesis and the role of STAT1-dependent mechanisms of IFN- β production during *Chlamydia* infection, our data showed that blocking IFNAR1 with antibody that will sterically hinder binding of type-1 IFNs to the IFNAR1/2 heterodimer ligand-binding site to disrupt induction of IFN- α transcription, had no effect on the induction of IFN- β transcription. We also showed that blocking IFNAR1 with antibody did not inhibit the ability of IFN- β to induce IFN- β gene expression in an autocrine/paracrine manner, thus proposing a

mechanism of IFN- β autoregulation that does not require IFNAR1, and likely involves a novel receptor for IFN- β (summarized in Fig. 11).

However, we must be cognizant of alternative interpretations of our data including the possibility that the IFNAR receptor complex may form alternate ligand binding sites that are not blocked by this specific antibody. In this regard, blocking IFNAR with this particular antibody would successfully block the IFN- β binding site required for induction of IFN- α , but it does not block a possible secondary site that IFN- β can bind to for synthesis of IFN- β . Alternatively, IFNAR2 may homodimerize or form heterodimers with some other known or unknown component to form a receptor complex that IFN- β can bind to specifically induce IFN- β gene expression via the type-1 IFN signaling pathway. In this regard, the paradigm holds true that the type-1 IFN signaling pathway is active in the synthesis of IFN- β ; however, the constitution of the IFNAR complex is different and its ability to bind IFN- β is not blocked by this particular antibody. Future experiments will further examine the role of IFNAR signaling in which we employ other mechanisms such as

si-RNA and/or derivation of OE cells from IFNAR knockout mice to ensure complete disruption of IFNAR signaling.

Acknowledgments

The authors thank Dr. Akira Moh (Indiana University-Purdue University, Indianapolis) for kindly providing matched WT and STAT1-deficient mice, from which OE cells were harvested for this study. We also thank Drs. David Nelson and Cheikh Seye for their critical review of this article.

Author Disclosure Statement

No competing financial interests exist.

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Received 17 January 2015/Accepted 8 June 2015